Investigations into the lymphocyte phenotypes and the presence of rheumatoid factor and antinuclear antibody in the peripheral blood of 515 dogs

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ABSTRACT: The levels of rheumatoid factor (RF), antinuclear antibody (ANA), and composition of peripheral lymphocyte subsets in 515 dogs were examined. Of these sample cases, 33 cases were diagnosed as immune-mediated fever that presented with high C-reactive protein (CRP), 31 cases were diagnosed with Hansen's Type 1 disc herniation and the remaining 415 cases were clinically healthy dogs, and served as controls. In the cases diagnosed with immune-mediated fever, 84% of the dogs tested positive to either RF or ANA (RF positive 60.6%; ANA positive 24.2%). By contrast, 16.2% of the healthy dogs were positive for either RF or ANA (RF positive 14.9%; ANA 1.3%). The CD4/CD8 ratio for peripheral lymphocyte was high for all analysed cases diagnosed with immune-mediated fever, and was significantly higher than those of healthy controls. These results indicate that the abnormal levels of lymphocytes may be an effective indicator for immune-mediated disease coupled to immune-mediated fever.

Keywords: $CD4^+/CD8^+$ ratio; immune-mediated disease; herniated disc; canine; C-reactive protein; lymphocyte

Pyrexia of unknown origin manifests frequently in dogs. In human medicine, pyrexia of unknown origin can be classed into three categories in accordance with the signs and symptoms. (1) Long-term pyrexia coupled with unspecific symptoms such as torpor, loss of appetite and weight loss that lasts for more than three weeks; (2) body temperature remains above the conventional body temperature by more than 0.83 over several measurements; (3) non-determinable symptoms observed in in-patient settings for a week or during a normal clinical analysis (Petersdorf and Beeson 1961; Vickery and Quinnell 1977; Bohnhorst et al. 2002). These standards cannot be applied to dogs directly; however, it can be considered as an effective guideline for eliminating infection as the cause of pyrexia. The cause of pyrexia of unknown origin has been reported in humans (Petersdorf and Beeson 1961; Jacoby and Swartz 1973) to be due to infection (40%), tumours (20%), immune system (15%), other diseases (20%) and unknown (5%). Dunn and Dunn (1998) examined 101 dogs presenting with pyrexia of unknown origin, and reported the causes to fall into six different groups, infection (16%), tumours (9.5%), immune system (22%), other diseases (11.5%), primary marrow disease (22%) and unknown (19%). However, large-scale investigations into the levels of RF and ANA in dogs have not yet been conducted. The lymphocyte fraction in the dog's immune system has been analysed using a series of monoclonal antibodies that detects canine cell surface antigens (Williams 1997). The similarities in the peripheral subset of lymphocytes in dogs and humans are becoming apparent (Moore et al. 1992; Faldyna et al. 2001), yet no reports have been conducted to investigate the lymphocyte composition in canine immune-mediated fever. The aim of the current study was to determine the differences in the levels of RF, ANA and phenotype subsets of peripheral lymphocytes of healthy controls and canine herniated disc cases.

MATERIAL AND METHODS

Animals

This study was conducted on 515 dogs (male 295, female 220). The total number of animals consisted of 31 dogs (male 21, female 11) diagnosed as suffering from herniated disc by CT scan, MRI and neurological examination; 33 dogs (male 20, female 11) grouped as suffering from immune-mediated disease under the definition of Bohnhorst et al. (2002), presenting with a high level of C-reactive protein (CRP). Of the dogs admitted to the clinic for prevention of filariasis, and presenting with normal signs under the general clinical examinations and body temperatures, 441 (male 254, female 197) were employed as healthy control subjects. The breeds of the dogs used in this experiment were as listed in Tables 1, 2 and 3. The actual measured body temperature values ranged between 39.9 °C and 40.5 °C.

The study was performed in accordance with the Japanese Regulations for Animal Welfare issued by The Ministry of Education, Culture, Sports, Sciences, and Technology of Japan.

C-reactive protein (CRP) measurement

Laser CRP-2 (Arrows, Osaka, Japan) was used to measure the C-reactive protein (CRP) level with immunonephelometry using 30 µl of serum. The measuring range was set between 0.05–20 mg/dl.

Rheumatoid factor and antinuclear antibody measurements

Rheumatoid factor (RF) was measured according to the manual of Canine Rheumatoid Factor Test Kit (Synbiotics Corporation, CA, USA). For antinuclear antibody (ANA) measurement tenfold and hundred-fold dilutions of serum samples were prepared. These dilutions were subsequently applied to the wells of immunofluorescence assay (IFA) slides (VMRD Inc. WA, USA) that had been smeared with cells and placed in the moisture chamber. The moisture chamber was shielded from light and the reaction was allowed to proceed for 30 min at 37 °C. The IFA slides were then removed from the chamber and the dilution sample on the slides discarded, and washed with cleaning fluid. The slides were then dried in air and placed in a chamber for application of secondary antibody and IgG antibody (selective for cats and dogs, VMRD Inc., WA, USA) to the reaction well on the slides. These were allowed to react for 30 min at 37 °C. After the incubation, the IFA slides were removed from the chamber, the dilution fluid was discarded and then the slides were washed with cleaning liquid. Finally, the slides were dried in air before observing the samples under a dark-field fluorescent microscope.

RF and ANA analyses were conducted by Monolis, Inc. (Tokyo, Japan), a clinical inspection body specialising in animals.

Flow cytometric analysis of peripheral blood

The lymphocyte compositions of the 22 healthy dogs, and 16 dogs with immune-mediated disease were analysed. The relative ratios of $CD3^+$ (T-cells), $CD4^+$ (helper T-cells), $CD8^+$ (Cytotoxic T-cells) and $CD21^+$ (B-cells) were obtained using a Accuri C6 flow cytometer (Accuri Cytometers, Inc., MI, USA) employing the canine-specific antibodies listed in Table 4. Blood samples of 200 µl were mixed with 2 µl of FACS lysis solution (Becton, Dickinson and Company, NJ, USA) and incubated for 15 min at room temperature. The peripheral white blood cells (WBC) were isolated by centrifugation (200 g) for 5 min, at room temperature. The WBC fraction was rinsed before a sufficient amount of antibodies (Serotec, Oxford, UK) were added. The samples were incubated in the dark for 15 min at room temperature. The suitable concentration of antibodies was calculated beforehand. Once the reaction was completed, 2 ml of phosphate buffer saline (PBS) solution was added to remove the excess primary antibodies. Finally, the cells were suspended in 500 µl of FACS FLOW (Becton, Dickinson and Company, NJ, USA) and kept at 4 °C until analysis. Accuri C6 flow cytometer and CFlow Plus software (Accuri Cytometers, Inc. MI, USA) were employed for sample analysis. The cell phenotypes of the peripheral lymphocytes were obtained by gating on the forward scatter versus side scatter dot plot obtained using the FACS Calibur. For each sample, 5 000 events in the lymphocyte gates were recorded from which the percentages of $CD3^+$, $CD4^+$, $CD8^+$ and $CD21^+$ were categorised according to the different lymphocyte surface markers over 5 000 events. Absolute values for lymphocyte subsets

were calculated using counts obtained from WBC analysis in combination with the flow cytometer. These procedures were conducted in accordance with Mori et al. (2008).

Neurological examination

Neurological examinations were conducted on subjects diagnosed with herniated disc. Examinations consisted of posture response (proprioception, placing reaction, hopping reaction, and extensor postural thrust), and spinal reflexes (patellar reflex, cranial tibial reflex, gastrocnemius reflex, flexion reflex, crossed-extension reflex, and panniculus reflex), presence of nociception and ability to self-urinate.

Diagnostic imaging

Computed tomographic imaging scans (1-mm thick) and magnetic resonance imaging scans (T1- and T2-weightedimages) were conducted under general anaesthesia. CT settings were 120 kVp, 80 mA, with a scan time of one second per slice. When evaluating the transverse CT images, the degree of attenuation of the intervertebral disc was measured in Hounsfield units (HU) in each image. The CT scan was conducted using Asteion (Toshiba Medical Systems, Tokyo, Japan). Magnetic resonance imaging was performed using a 0.2 T Vet-MR (Esaote S.p.A, Genova, Italy).

Statistical analyses

The data values feature mean ± standard deviation (S.D). The statistical significance of the values was determined using the Mann-Whitney *U*-test, and the statistical analyses were conducted using StatMate III for Windows (Atms, Tokyo, Japan). Values with $P < 0.001$ were considered statistically significant.

RESULTS

Rheumatoid factor (RF)

The numbers of dogs with positive values for RF were as follows, 66 of 451 healthy dogs (14.6%), 20 of 33 with immune-mediated disease (60.6%), and18 of 30 with herniated disc (58.1%). The numbers of positive results were particularly high for Dachshund and Chihuahua breeds, with 27 of 168 (16.1%) and 10 of 48 (20.8%), respectively. The results obtained for subjects with immune-mediated disease and herniated disc, alongside the healthy controls are displayed in Tables 1–3.

Antinuclear antibody (ANA)

Antinuclear antibody positivity was observed in six out of 451 healthy dogs (1.3%), eght out of 33 dogs with immune-mediated disease (24.2%) and one out of 31 with herniated disc (3.2%). The results obtained for subjects with immune-mediated disease and herniated disc, alongside the healthy controls are shown in Tables 1–3.

C-reactive protein (CRP)

The mean ± S.D. for subjects with immune-mediated disease and herniated disc were 6.9 ± 6.7 and 1.2 ± 0.9, respectively. The results obtained are shown in Table 2 and 3 in according to breed.

Flow cytometric analysis of peripheral blood

The flow cytometric results were as follows. The subsets for healthy control subjects were: $CD3^+$ lymphocytes 83.6 ± 3.4%; $CD21^+(CD3^-)$ lymphocytes 8.7 ± 3.3%; $CD3^-CD21^-$ lymphocytes 7.7 ± 2.8%; $CD4^+(CD8^-)$ lymphocytes 49.2 ± 3.5%; $CD8^+(CD4^-)$ lymphocytes 27.4 ± 3.7% and 1.8 ± 0.2 for $CD4^+/CD8^+$ ratio. In dogs with immune-mediated disease, $CD3^+$ lymphocytes 77.1 ± 5.7%; $CD21^+(CD3^-)$ lymphocytes 7.5 ± 3.4%; $CD3^-CD21^-$ lymphocytes 15.5 ± 4.7%; $CD4^+(CD8^-)$ lymphocytes 56.4 ± 7.5%; $CD8^+(CD4^-)$ lymphocytes 15.7 ± 4.2% and 3.9 ± 1.4 for $CD4^+/CD8^+$ ratio. The expression of $CD3^-CD21^-$lymphocytes, $CD4^+(CD8^-)$ lymphocytes and $CD4^+/CD8^+$ ratio were found to be significantly higher in dogs with immune-mediated disease compared to the healthy subjects ($P < 0.001$). By contrast, the values for $CD3^+$ lymphocytes, $CD21^+(CD3^-)$ lymphocytes and $CD8^+(CD4^-)$ lymphocytes were found to be significantly lower in the immune-mediated disease group compared to those of the healthy controls ($P < 0.001$) (Table 5).

Table 1. Demographics of the study groups

Breed	Number	Female	Male	Age[a]	RF (rate, %)	ANA (rate, %)
All Breeds	451	197	254	5.1 ± 2.9	66 (14.6)	6 (1.3)
Dachshund	168	76	92	6.0 ± 2.6	27 (16.1)	3 (1.8)
Chihuahua	48	20	28	3.7 ± 1.7	10 (20.8)	0
Toy Poodlle	33	13	20	3.0 ± 2.7	4 (12.1)	1 (3.0)
Shihtzu	21	9	12	5.5 ± 3.6	4 (19.0)	0
Shiba inu	21	10	11	4.3 ± 2.1	0	0
Cavalier King Charles Spaniel	14	6	8	5.3 ± 2.9	1 (7.1)	0
Yorkshire Terrier	13	7	6	5.7 ± 3.5	0	0
Papillon	12	4	8	5.6 ± 3.7	2 (16.7)	0
Welsh Corgi Penbroke	12	2	10	6.3 ± 2.5	1 (8.3)	1 (8.3)
Miniature Schnauzer	9	4	5	5.4 ± 2.8	3 (33.3)	0
Pomeranian	9	3	6	3.1 ± 1.8	1 (11.1)	0
Beagle	7	2	5	4.7 ± 2.5	2 (28.6)	0
West Highland White Terrier	6	4	2	5.8 ± 1.3	1 (16.7)	0
Maltese	5	2	3	5.4 ± 3.4	0	0
Border Collie	5	3	2	5.2 ± 3.4	0	0
Jack Russell Terrier	5	2	3	6.0 ± 5.7	2 (40.0)	0
Italian Greyhound	4	2	2	3.7 ± 2.2	0	0
Gorden Retriever	4	2	2	8.2 ± 3.5	0	0
Labrador Retriever	4	1	3	4.0 ± 0.8	0	0
Pug	3	1	2	2.0 ± 1.0	0	0
Miniature Pinscher	3	2	1	3.7 ± 1.2	0	1 (33.3)
Shetland Sheepdog	3	2	1	6.3 ± 4.6	1 (33.3)	0
Saluki	2	1	1	3.5 ± 2.1	2 (100)	0
Basenji	2	1	1	6.0 ± 1.4	0	0
French Bulldog	2	0	2	3.5 ± 3.5	2 (100)	0
Boxer	2	0	2	0.6 ± 0.2	0	0
Pekingese	1	0	1	2	0	0
Boston Terrier	1	1	0	2	0	0
Whippet	1	0	1	4	0	0
American Cocker Spaniel	1	1	0	2	0	0
Great Pyrenees	1	1	0	7	0	0
Australian Silky Terrier	1	0	1	2	0	0
Parson Russell Terrier	1	0	1	7	0	0
Kooikerhondji	1	0	1	5	0	0
Flat Coated Retriever	1	0	1	2	0	0
German Shepherd Dog	1	1	0	2	0	0
Mix Breed	24	15	9	4.8 ± 4.3	4 (16.7)	0

RF = rheumatoid factor; ANA = antinuclear antibody
[a]age is expressed as mean ± S.D.

Table 2. Summary of the disease cases

Breed	Number	Female	Male	Age[a]	RF (rate, %)	ANA (rate, %)	CRP[b]
All Breeds	33	12	21	6.1 ± 2.0	20 (60.6)	8 (24.2)	6.9±6.7
Dachshund	26	9	17	6.3 ± 1.8	14 (53.8)	7 (26.9)	5.6±5.8
Papillon	1	1	0	6	1 (100)	0	6
West Highland White Terrier	1	1	0	10	1 (100)	0	4.5
Chihuahua	1	1	0	4	1 (100)	0	1.6
French Bulldog	1	0	1	7	1 (100)	0	7.8
Shihtzu	1	0	1	4	1 (100)	0	20
Toy Poodlle	1	0	1	2	0	1 (100)	20
Welsh Corgi Penbroke	1	0	1	4	1 (100%)	0	20

Disease: immune-mediated fever that presented with high C-reactive protein (CRP)
RF = rheumatoid factor; ANA = antinuclear antibody
[a]age is expressed as mean ± S.D.
[b]CRP is expressed as mean ± S.D.

Table 3. Summary of the intervertebral disc herniation data

Breed	Number	Female	Male	Age[a]	RF (rate, %)	ANA (rate, %)	CRP[b]
All Breeds	31	11	20	7.6 ± 3.0	18 (58.1)	1 (3.2)	1.2 ± 0.9
Dachshund	26	11	15	7.7 ± 3.1	13 (50)	1 (3.8)	1.2 ± 1
French Bulldog	2	0	2	8 ± 2.8	2 (100)	0	1.5 ± 0
Welsh Corgi Penbroke	2	0	2	8.5 ± 2.1	2 (100)	0	1.25 ± 0.1
Toy Poodlle	1	0	1	4	1 (100)	0	2.6

RF = rheumatoid factor; ANA = antinuclear antibody
[a]age is expressed as mean ± S.D.
[b]CRP (C-reactive protein) is expressed as mean ± S.D.

Table 4. Monoclonal antibodies used in the present study

Phenotype	Specificity	Host	Isotype	Conjugate	Clone
CD21	B-cells	mouse	IgG1	RPE	CA2.1D6
CD3	T-cells	mouse	IgG1	FITC	CA17.2A12
CD4	helper T-cells	rat	IgG2a	FITC	YKIX302.9
CD8	cytotoxic T-cells	rat	IgG1	RPE	YCATE55.9

RPE = Rhodophyceae phycoerythrin; FITC = fluorescein isothiocyanate isomer I

Table 5. T lymphocytes phenotypes in canine disease blood (n = 16) compared with canine control blood (n = 22)

Phenotype	Control	Disease	Mann-Whitney's U-test
$CD3^+$ lymphocytes	83.6 ± 3.4	77.1 ± 5.7	$P < 0.001$
$CD21^+$ ($CD3^-$) lymphocytes	8.7 ± 3.3	7.5 ± 3.4	$P < 0.001$
$CD3^-$ $CD21^-$ lymphocytes	7.7 ± 2.8	15.5 ± 4.7	$P < 0.001$
$CD4^+$ ($CD8^-$) lymphocytes	49.2 ± 3.5	56.4 ± 7.5	$P < 0.001$
$CD8^+$ ($CD4^-$) lymphocytes	27.4 ± 3.7	15.7 ± 4.2	$P < 0.001$
$CD4^+/CD8^+$ ratio	1.8 ± 0.2	3.9 ± 1.4	$P < 0.001$

Disease: immune-mediated fever that presented with high C-reactive protein
All values are expressed as mean ± S.D.
The differences in values with the controls were evaluated using Mann-Whitney's U-test

DISCUSSION

Pyrexia of unknown origin in dogs has been defined as a disease that, in addition to the definitions for human disease, features body temperatures of over 40 °C (Dunn and Dunn 1998). In the current study, immune-mediated disease was defined as cases that displayed high levels of CRP in accordance with the specifications of Bohnhorst et al. (2002).

Organisms have immune mechanisms that function to eliminate the foreign materials that invade the body. Autoimmunity is a phenomenon whereby lymphocytes that act as the "control centre", attack the body's own cells. When this presents as a disease state, it is referred to as immune-mediated disease. It is a chronic condition that is difficult to cure once the "control centre" is programmed with the incorrect information. The symptoms of immune-mediated disease are thought to manifest as a sum of the immune responses against the various antigens present in the body.

The mechanisms of immune responses involving cytokines as the key players have been investigated; however, to identify the mechanisms of immune-mediated diseases in more depth, an understanding of auto immune responses that involve T-lymphocytes cannot be neglected.

Canine immune-mediated disease is diagnosed by a process of elimination (diagnosis of exclusion). The evaluation of synovial fluid, RF, ANA are used as parameters, but since these are non-specific, they cannot be used as a definitive diagnosis (Bohnhorst et al. 2002). Bohnhorst et al. (2002) reported that seven out of 20 (35%) dogs with immune-mediated fever were positive for ANA, and the remaining 13, except two (15%), to have been negative for RF. The percentages of dogs with immune-mediated arthritis that presented as positive for RF are reported to range between 25 to 75% (Davidson 2003). The positive rate for ANA and RF were 24.2% and 60.6%, respectively, in the immune-mediated disease subjects, and 1.3% and 14.6%, respectively, for healthy control subjects. The results obtained from the current study are similar to previous studies, in that they also did not allow definitive disease diagnosis.

A marked percentage, 58.1%, of herniated disc subjects displayed positive for RF suggesting that positive RF values correlate with an increased probability of contracting disc herniation. Future research using an increased cohort size is required to investigate the correlation between RF levels and incidence of herniated disc.

A marked increase in serum CRP concentrations is known to occur as a result of inflammation or tissue destruction (Ceron et al. 2005). In human medicine, CRP levels, used as a prediction marker for some cases of neoplasia (Shimada et al. 2003), and to determine the prognosis of myocardial infarction and stroke cases (Winbeck et al. 2002; Clearfield 2005), are used as an inflammation marker to determine the severity of the disease state. Recent investigations into canine CRP levels have reported elevated levels in a number of disease states including irritable bowel syndrome (IBD) (Jergens et al. 2003) and autoimmune haemolytic anaemia (AIHA) (Tecles et al. 2005). A marked increase in CRP levels was noted in all of the canine immune-mediated arthritis cases analysed in a recent study (Ohno et al. 2006). A high level was also observed in this study with an average of 6.9 ± 6.7%. Elevated CRP levels are often observed in numerous disease states coupled with inflammation; therefore, it cannot be adopted as a specific marker except to evaluate the therapeutic response of the subject.

To characterise the surface markers, flow cytometry was employed. A flow cytometer can be one of two sorts depending on its functions, either a cell sorter or a cell analyser. The former has the ability to collect the subsets of cells that it has been programmed to analyse and measure at the same time, whereas the latter can carry out the measurement only. A cell analyser was used in this study.

CD3 is a surface marker that is displayed on mature T-lymphocytes. These T-lymphocytes can be further divided into subsets that are $CD4^+(CD8^-)$ helper T-lymphocytes, and $CD8^+(CD4^-)$ cytotoxic T-lymphocytes. Surprisingly, the ratios of these subsets in dogs have been found to be extremely similar to those found in humans (Moore et al. 1992; Faldyna et al. 2001). Harris et al. (1992) have reported the CD4/CD8 ratios in healthy human subjects to be 1.7, while the results of the current study showed the $CD4^+/CD8^+$ ratio to be 1.8 ± 0.2, confirming the canine subsets of peripheral lymphocytes to be similar to those of humans. The $CD4^+/CD8^+$ ratio of humans presenting with immune-mediated disease was reported to be significantly higher than that of the healthy individuals (Gulizia et al. 1993). The $CD4^+/CD8^+$ ratio of all of the 16 cases of immune-mediated disease that were examined here displayed high levels, with an average of 3.9 ± 1.4. Statistical testing revealed the

values obtained from the subjects with the disease to be significantly higher than those of healthy controls ($P < 0.001$). These results indicated that the $CD4^+/CD8^+$ ratio could be a novel diagnostic parameter for determining the presence of immune-mediated disease.

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Received: 2012–07–18
Accepted after corrections: 2012–09–30

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