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Changes in lymphocyte function and subset counts in cats with spontaneous chronic kidney disease

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ABSTRACT: Chronic kidney disease alters the immune response in humans and dogs and it is thought to be one of the causes of immunosuppression in cats. Haematological, biochemical, and immunological examinations were performed on blood samples obtained from 20 cats with chronic kidney disease, and were compared with the results of 18 healthy cats. In cats with chronic kidney disease, statistically significantly lower numbers of total lymphocytes ($P < 0.01$), especially T lymphocytes ($P < 0.001$), were observed. When the results were compared with the lymphocyte transformation test in healthy cats, decreased responses to phytohaemaglutinin, concanavalin A, and pokeweed mitogen were found in cats with chronic kidney disease. Our results document an alteration of counts and activity of lymphocytes in cats with chronic kidney disease. Thus, higher susceptibility to infection may be expected in these patients.

Keywords: uraemia; immunosuppression; lymphopenia; mitogens; flow cytometry

Chronic kidney disease (CKD) is a common problem of aging dogs and cats. It is thought to be a cause of secondary immunosuppression of non-infectious origin. This is well documented in human medicine where infection is a severe and life-threatening complication of CKD. Infection is the second most common cause of death in people with end-stage renal disease (Girndt et al. 1999; Sarnak and Jaber 2000). This is largely due to the impaired immune response in uraemia (Girndt et al. 2001). Both humoral and cell mediated immunity are disturbed (Cohen and Horl 2012; Vaziri et al. 2012). Common findings in people with CKD are lymphopenia and altered lymphocyte function (Matsumoto et al. 1995; Meier et al. 2002; Yoon et al. 2006). In dogs with CKD, we have documented lymphopenia with more considerable reductions in B cells, Tc cells, NK cells and a depressed lymphocyte response to concanavalin A and pokeweed mitogen, neutrophilia and increased lysozyme levels (Kralova et al. 2009; Kralova et al. 2010). Silva et al. (2013) described an impairment of neutrophil function due to the presence of oxidative stress and acceleration of neutrophil apoptosis. In feline medicine, only a few reports exist dealing with the immune response in cats with chronic kidney disease. Keegan and Webb (2010) reported increased oxidative stress and lower antioxidant capacity in CKD cats.

To our knowledge, there is no previous report describing the changes in lymphocyte number and function in cats with chronic kidney disease. The purpose of this study was to compare the lymphocyte numbers and their function in clinically healthy cats and cats with CKD.

MATERIAL AND METHODS

Animals. Twenty cats diagnosed with chronic kidney disease were included in the study. These cats were patients of the Clinic of Dog and Cat Diseases, Faculty of Veterinary Medicine, UVPS

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Brno, Czech Republic. The diagnosis of CKD was based on history, clinical findings, haematological and biochemical examinations, urinalysis and ultrasonography of the urinary tract. The presence of persistent azotaemia (serum creatinine level above the laboratory reference value of 180 μmol/l) and poor renal concentrating ability were consistent findings. Only cats negative for feline immunodeficiency virus and feline leukemia virus infections were included in the study. Cats with chronic renal failure and another disease associated with secondary immunosuppression (e.g. diabetes mellitus, neoplasia) and cats treated with immunosuppressive drugs were excluded from the study.

The group comprised 11 females (all neutered) and nine males (six neutered) of various breeds (17 domestic short-haired cats, two Persian cats, one Siamese cat). Average age was 11.9 ± 3.6 years (ranging from six to 20 years); in one cat from a cat shelter, the age was unknown. For further evaluation, cats were categorised into two subgroups, according to the severity of clinical signs. The first group comprised eight cats (four males and four females, average age 11.7 ± 3.0 years) presented with clinical symptoms and/or physical examination findings compatible with uremic syndrome (inappetence/anorexia, lethargy, polyuria/polydipsia, vomiting, weight loss). Most of these animals were not dehydrated and they were not judged to be in need of fluid therapy. Dehydrated cats responded well to fluid and supportive therapy and survived more than one month. This subgroup was referred to as uremic cats. The remaining twelve cats (five males and seven females, average age 12.1 ± 3.9 years) were considered to be in end-stage renal disease. These animals were presented with the most severe clinical symptoms and failed to respond to treatment, surviving less than 21 days following the diagnosis. These patients succumbed to disease or were euthanized.

Results from eighteen healthy cats (eight males and 10 females, average age 7.5 ± 2.5 years) from a private households and from the Veterinary Research Institute databases were used as control data for analysis.

Immunological tests. Total leukocyte counts were determined using the Digicell 500 cell counter (Contraves AG, Zurich, Switzerland); differential leukocyte counts were enumerated from blood smears stained according with May-Grunwald and Giemsa-Romanowski (Torrance 2000).

Cells were stained by the indirect unicolour technique. Fifty microlitres of blood were incubated with monoclonal antibodies for 15 min at laboratory temperature. Erythrocytes were lysed with 3 ml of a haemolytic solution (8.36 g NH_4Cl, 1 g $KHCO_3$ and 0.037 g EDTA per 1 l of distilled water). Mouse anti-cat monoclonal antibodies CD4 (FE.17B12), CD5 (FE1.1B11), CD8 (FE1.10E9) and CD21 (FE2.9F6) kindly provided by Dr. P.F. Moore (University of California, Davis, CA, USA) were used. In the next step, secondary goat anti-mouse IgG1-FITC or IgG2-R-PE antibodies (Southern Biotech, Birmingham, AL, USA) were added followed by incubation for 20 min at 4 °C. Propidium iodide was used to stain the DNA of dead and damaged cells and to exclude such events from the analysis. Data were acquired on a standard FACSCalibur flow cytometer (Becton Dickinson, Mountain View, Alberta) operated by the CELLQuest software. Gating of the lymphocyte population was based on forward angle and right angle scatter signals. In each sample, 10 000 cells were measured.

The proliferation of lymphocytes was determined using the mitogen-driven lymphocyte transformation test. Heparinised blood (0.5 ml of full blood with 50 μl of heparin) was mixed with 0.25 ml of 5% foetal calf serum and 4.25 ml of RPMI 1640 medium. Twenty ml of mitogens – phytohaemaglutinin (PHA, 40 μg/ml, Murex Biotech, Ltd., Kent, United Kingdom), concanavalin (ConA, 10 μg/ml, Pharmacia Biotech, AB, Sweden) and pokeweed mitogen (PWM, 10 μg/ml, Sigma-Aldrich, Chemie) were pipetted in triplicates into wells of microtitre plates (Gama Group, Ceske Budejovice, Czech Republic). Cell suspensions of blood cells (200 μl) were then added to each well, including triplicate wells without mitogen. The microplates were incubated for three days at 37 °C and with 5% CO_2. ^{3}H-thymidine (50 μl) was added 20 h before the end of incubation. The incorporation of ^{3}H-thymidine was measured with a liquid scintillation counter (TopCount NXT, Packard Bioscience Instrument Company, Meriden, Connecticut, USA). The results were expressed as counts per minute (CPM) in stimulated samples versus CPM in non-stimulated controls.

Data analysis. Statistics were calculated with MS-Excel 6.0 (mean ± SD) and Graph Pad Prism Software (inter-group differences). Statistical differences between groups were estimated with the unpaired non-parametric Mann-Whitney test. Differences with $P < 0.05$, $P < 0.01$, and $P < 0.001$

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were interpreted as significant, highly significant and very highly significant, respectively. Correlations between parameters were calculated using the Spearman test.

RESULTS

Total and differential leukocyte counts

Total and differential leukocyte counts are summarised in Table 1. In cats with chronic kidney disease, we found significantly more neutrophils whereas the number of lymphocytes was highly significantly lower in comparison with healthy cats. These changes were more pronounced in the subgroup of cats with end-stage renal disease. In this subgroup, six lymphopenic cats (lymphocytes less than 1500 × 10^6/l) were present, whereas in the subgroup of uremic cats, there was only one. Eosinophil counts in the group of healthy cats were significantly higher as compared to the group of cats with CKD.

Lymphocyte subsets

In CKD cats, a statistically significant decrease of $CD3^+$ T lymphocyte counts (both $CD8^+$ Tc and $CD4^+$ Th subpopulations) was found. This decrease was expressed especially in the cats with end-stage renal disease. The changes of $CD21^+$ B lymphocyte counts were not significant. Data are summarised in Table 2.

Table 1. Total and differential leukocyte counts (10^6/l) in cats with CKD and healthy control group (mean ± SD)

Parameter	Control cats (n = 18)	CKD cats (n = 20)	Uremic cats (n = 8)	End-stage cats (n = 12)
Leukocytes	10 944 ± 4 273	11 150 ± 6 219	9 453 ± 5 035	12 281 ± 6 248
Neutrophils	4 734 ± 2 989	8 584 ± 6 512*	5 900 ± 4 007	10 372 ± 6 863**
Lymphocytes	4 785 ± 2 043	2 146 ± 1 768**	3 069 ± 1 661	1 529 ± 1 439***
Monocytes	363 ± 193	311 ± 226	331 ± 270	297 ± 160
Eosinophils	1 028 ± 589	137 ± 162***	219 ± 172**	83 ± 110***

*P < 0.05, **P < 0.01, ***P < 0.001

Table 2. Lymphocyte subsets (10^6/l) in cats with CKD and healthy control group (mean ± SD)

Lymphocyte subset	CD molecules	Control cats (n = 18)	CKD cats (n = 20)	Uremic cats (n = 8)	End-stage cats (n = 12)
T lymphocytes	$CD5^+$	3092 ± 1534	1268 ± 1162***	1575 ± 621	1071 ± 137***
T helper	$CD4^+$	1570 ± 751	615 ± 610***	682 ± 358*	573 ± 706***
T cytotoxic	$CD8^+$	1338 ± 784	514 ± 502***	695 ± 346	398 ± 543***
B lymphocytes	$CD21^+$	1422 ± 1018	862 ± 838	1416 ± 941	509 ± 364*

*P < 0.05, **P < 0.01, ***P < 0.001

Table 3. Proliferative activity of lymphocytes non-stimulated and stimulated with different mitogens (phytohaemaglutinin, concanavalin A, pokeweed mitogen), expressed as counts per minute (CPM)

Parameter	Control cats (n = 18)	CKD cats (n = 20)	Group A (n = 8)	Group B (n = 12)
Non-stimulated lymphocytes	500 ± 506	181 ± 218*	207 ± 245*	164 ± 172*
Phytohaemaglutinin	2 995 ± 2 942	582 ± 833***	989 ± 928*	311 ± 517***
Concanavalin A	12 594 ± 12 728	3 333 ± 3 132**	5 314 ± 3 048*	2 013 ± 2 052***
Pokeweed mitogen	3 911 ± 2 926	874 ± 890***	1 227 ± 874**	639 ± 737***

*P < 0.05, **P < 0.01, ***P < 0.001

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Table 4. Correlation between the number of B lymphocytes, Tc lymphocytes and the levels of creatinine, urea and phosphorus in cats with chronic renal failure

Parameter	B lymphocytes	Tc lymphocytes
Creatinine	$r = -0.4877$, $P = 0.0342$	$r = -0.4930$, $P = 0.0320$
Urea	$r = -0.5333$, $P = 0.0187$	$r = -0.4632$, $P = 0.0458$
Phosphorus	$r = -0.5807$, $P = 0.0091$	$r = -0.5105$, $P = 0.0255$

Lymphocyte transformation test

The activity of non-stimulated lymphocytes was found to be significantly lower in CKD cats compared to the clinical healthy group ($P < 0.05$). Further, in cats with chronic kidney disease, a significantly lower response to all used mitogens compared to the clinical healthy group was observed. The changes were more substantial in the end-stage group ($P < 0.001$; see Table 3).

Correlation between parameters

There was a significant negative correlation between the number of lymphocytes and the creatinine level ($r = -0.4677$; $P = 0.0355$), the urea level ($r = -0.4722$; $P = 0.0355$) and the phosphorus level ($r = -0.5293$; $P = 0.0164$). The number of eosinophils showed a negative correlation with the urea level ($r = -0.4871$; $P = 0.0294$) and the phosphorus level ($r = -0.5293$; $P = 0.0164$). The number of B cells and Tc cells showed a negative correlation with the levels of creatinine, urea and phosphorus (Table 4). The lymphocyte activity after ConA and PWM stimulation showed a statistically significant negative correlation with the level of phosphorus (ConA: $r = -0.4632$; $P = 0.0397$, PWM: $r = -0.4647$; $P = 0.0390$).

DISCUSSION

While deaths in the human population owing to bacterial infections have generally fallen, they remain the second most common cause of death in patients with end-stage renal disease (Girndt et al. 1999; Sarnak et al. 2000). This is largely due to the impaired immune response in uraemia (Girndt et al. 1999; Girndt et al. 2001; Meier et al. 2002), which is caused by decreased granulocyte and monocyte/macrophage phagocytic function (Massry and Smogorzewski 2001), depletion of antigen-presenting cells and impairment of their antigen presenting capacity (Agrawal et al. 2010), reduced numbers and antibody-producing capacity of B lymphocytes (Smogorzewski and Massry 2001; Pahl et al. 2010), increased T cell and apoptosis leading to depletion of naive and central memory $CD4^+$ and $CD8^+$ T lymphocytes, and impaired cell-mediated immunity (Matsumoto et al. 1995; Moser et al. 2003; Vaziri et al. 2012).

Lymphopenia is a typical finding in patients with chronic kidney disease in humans, dogs, and cats (Chew et al. 1983; DiBartola et al. 1987; DiBartola et al. 1989; Matsumoto et al. 1995; Yoon et al. 2006). Our study confirmed decreased lymphocyte numbers in CKD cats in comparison with healthy cats. Lymphopenia was more frequent in cats with end-stage renal disease. The decrease of lymphocytes was more evident in T cells whereas the changes in B lymphocyte subpopulations were not significant. This is consistent with results of previous studies in humans (Alvarez-Lara et al. 2004; Soriano et al. 2005). In a previous study of dogs with chronic kidney disease, a more considerable reduction of Tc cells demonstrated by an increase in the Th/Tc ratio was found (Kralova et al. 2010). In most human studies, the depression in T cell number is proportionally distributed between Th and Tc lymphocytes and does not lead to a significant change in the Th/Tc ratio (Matsumoto et al. 1995; Meier et al. 2002). This finding was also observed in our study in cats.

According to the most recent research on uraemia in humans, lymphopenia is a consequence of accelerated apoptosis of lymphocytes. This may result from a higher expression of the Fas (CD95) molecule or a lower expression of Bcl-2 (Matsumoto et al. 1995; Meier et al. 2002). Increased expression of the Fas molecule, the mediator of T cell apoptosis, has frequently been observed in uremic lymphocytes. Bcl-2 is a member of the Bcl-2 family with anti-apoptotic effects and is capable of protecting lymphocytes against a variety of apoptotic signals. A significantly decreased expression of Bcl-2 has been observed in $CD4^+$, $CD8^+$ and $CD19^+$ lymphocytes obtained from uremic patients (Fernandez-Fresnedo et al. 2000).

In a recent study, Hendrikx et al. (2009) demonstrated increased apoptosis and a marked reduction in Treg cells ($CD4^+/CD25^+/FoxP3^+$) in end-stage renal disease patients. Incubation of isolated Treg cells from normal subjects with uremic serum *ex*

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vivo lowered the number and reduced the suppressive capacity of these cells, pointing to the deleterious effect of the uremic milieu on these cells. Given the critical role of Treg cells in mitigating inflammation, Treg cell deficiency and dysfunction in end-stage renal disease patients must contribute to the prevailing systemic inflammation.

Lymphopenia *per se* does not explain the immune dysfunction described in uremic patients. T cells represent a major component of the adaptive immune system and play a central role in cell-mediated immunity. Th lymphocytes participate in the activation of cytotoxic T cells and macrophages, maturation of B cells and their antibody production, recruitment of polymorphonuclear leukocytes, eosinophils and basophils to the loci of infection or in amplification of microbicidal activity of macrophages. Tc lymphocytes can destroy virally infected cells and tumour cells (Vaziri et al. 2012). These immune activities are determined by the different cytokine profile produced by Th lymphocytes (Th1 × Th2). Thus, the decreased lymphocyte counts may lead to production of altered amounts of cytokines contributing to an altered immune response.

The significantly lower response to mitogens in cats with chronic renal failure, especially in cats with severe clinical signs, is an important finding. This is consistent with previous reports from human medicine (Nakhla and Goggin 1973; Raska et al. 1983) and our findings in dogs (Kralova et al. 2010). The extent of the diminished response to phytohaemaglutinin may correlate with the fall in the number of T lymphocytes as phytohaemaglutinin is thought to be mainly a T cell mitogen. From the clinical point of view, the correlation between the number of lymphocytes and their activity is very significant. The finding of lymphopenia may predict an altered immune response.

In the present study, patients were divided into groups for further evaluation according to the extent of clinical signs at the time of the diagnosis and the response to therapy. This system was used earlier in cats with chronic kidney disease (Elliott and Barber 1998). In our opinion, this classification is more suitable for the evaluation of the patients in this study than the commonly used International Renal Interest Society (IRIS) system, where the level of creatinine is the main criterion (www.iris-kidney.com/guidelines/en/staging_ckd.shtml). According to the IRIS system, 17 of 20 CKD cats in our study would be classified in Stage 4 (creatinine > 440 mmol/l) and only three cats in Stage 3. In these three cats, the creatinine level ranged between 291.2 and 412.7 mmol/l. Thus, a description which takes into account the severity of the symptoms and the response to therapy is better for patient characterisation than the assessment of creatinine level as a single parameter.

Limitations of this study include the fact that control cats were younger than CKD cats. The age of animals may influence the immune system. Campbell et al. (2004) reported significantly lower leukocyte, lymphocyte and eosinophil counts and absolute counts of T cells, B cells and natural killer cells in older cats. Chronic kidney disease is a condition typical of older and geriatric patients. In our CKD group, 13 of 20 cats were older than 11 years (with the maximum of 20 years). In this age group, it is very difficult to find an animal that is completely healthy and suitable for the group of control animals. Despite our efforts, we were not able to obtain enough healthy geriatric cats, so we shifted the average age of control cats closer to the average age of CKD cats. In a study of CKD dogs, similar to the present work, we found disturbances in lymphocyte counts and function comparable to results in cats with chronic kidney disease (Kralova et al. 2010). In that study, the group of control dogs was age-matched to dogs with CKD. In our opinion, the influence of the age difference between healthy control and CKD cats is negligible and changes in the immune response in CKD cats are attributable to chronic kidney disease.

The group of healthy cats harboured significantly higher levels of eosinophils than CKD cats but these numbers were also higher than usual counts reported in healthy animals (Raskin et al. 2004). This might be linked with the fact that the majority of healthy cats were from cat shelters. Eosinophil counts in cats with CKD were in the physiological range.

In conclusion, lymphopenia and impaired lymphocyte activities are typical of cats with chronic kidney disease. The decrease in lymphocyte numbers was more evident in T cells and the changes are more considerable in cats with end-stage renal disease. This is consistent with previous reports on humans and dogs. As T cells (especially Th) play a central role in regulation of the immune response, our results indicate a disturbed immune response in cats with chronic kidney disease. Thus, higher susceptibility to infection may be expected in these patients.

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