Introduction

Chronic renal failure (CRF) is a common problem of aging dogs and cats. Renal failure is defined as a loss of 3/4 of functioning nephrons and it is associated with various clinical signs. The most common is polyuria and polydipsia due to loss of concentrating ability. Gastrointestinal complications (inapetence, anorexia, vomiting, diarrhea, weight loss) are very common, and they are usually the first signs that prompt owners to visit a veterinarian. Neurological abnormalities associated with CRF are very common and include dullness, lethargy, tremors, seizures, stupor, and coma; however, the severity of these clinical signs may vary. The patients can be presented at various stages of the disease, ranging from subclinical (detected by laboratory tests only — the presence of azotemia and inadequate urinary concentrating ability), mild (vomiting, weight loss, mild neurological signs) to severe azotemia (end stage, where the homeostasis is so disturbed, that it is incompatible with life). Chronic renal failure is associated with many changes in laboratory tests. The presence of azotemia and hyperphosphatemia is typical, anemia (nonregenerative, normochromic, and normocytic) is also commonly found (1).

Chronic renal failure causes secondary immunosuppression. This is well-documented in humans who have chronic kidney disease, where infection is a severe, life threatening complication (2–4). Chronic renal failure is often complicated by lymphopenia, and the CD4+ /CD8+ ratio may be unchanged or diminished. The depressed proliferative responses to the T-cell mitogens phytohemaglutinin (PHA) and concanavalin A (ConA) may be, in part, responsible for the uremic immunodeficiency (5–8). Similar results have been found in uremic rats (9,10).

Changes in lymphocyte function and subsets in dogs with naturally occurring chronic renal failure

Simona Kralova, Lenka Leva, Miroslav Toman

Abstract

Chronic renal failure (CRF) causes immunosuppression in humans and is thought to be one of the causes of noninfectious secondary immunosuppression in dogs. Hematological, biochemical, and immunological examinations were performed on blood samples obtained from dogs in various stages of CRF. The number of dogs with lymphopenia increased with the progression of clinical signs. All main subsets of lymphocytes were decreased, but more considerable reduction was detected in B-cells, Tc-cells, and NK cells. Depressed lymphocyte response to concanavalin A and pokeweed mitogen was found in dogs with severe clinical signs and lymphopenia. Our results, showing impaired immunological functions, are similar to results obtained from uremic humans, suggesting that infection may be an important complication in dogs with CRF.

Résumé

La défaillance rénale chronique (CRF) entraîne une immunosuppression chez l’humain et on croit qu’elle serait une des causes de l’immunosuppression secondaire non-infectieuse chez les chiens. Des examens hématologiques, biochimiques et immunologiques ont été effectués sur des échantillons sanguins obtenus de chiens avec des stades différents de CRF. Le nombre de chiens avec lymphopénie a augmenté avec la progression des signes cliniques. Tous les différents sous-groupes principaux de lymphocytes étaient diminués, mais une réduction plus importante était détectée avec les cellules B, les cellules Tc et les cellules NK. Une réponse lymphocytaire diminuée à la concanavaline A et au mitogène de la phytoïlate a été notée chez les chiens avec des signes cliniques sévères et une lymphopénie. Nos résultats, qui montrent une atteinte des fonctions immunologiques, sont similaires aux résultats obtenus d’humains urémiques, ce qui suggère qu’une infection pourrait être une complication importante chez les chiens souffrant de CRF.

(Traduit par Docteur Serge Messier)
### Materials and methods

**Animals**

Forty-five dogs with diagnosed chronic renal failure were assessed in the study. These dogs were patients of the Clinic of Dog and Cat Diseases, Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic and were presented to the clinic from May 2005 to June 2007. Dogs were diagnosed with CRF by the presence of persistent azotemia (serum creatinine level above the laboratory reference range of 125 μmol/L) in conjunction with poor urinary concentrating ability. Hematological examinations, standard biochemistry profiling, and urinanalyses were performed in all dogs, while the ultrasonographic examination of kidneys was carried out in 36 dogs. In cases where a differentiation of acute and chronic renal failure was not possible, histopathological examination of renal tissue was performed. Animals with signs of significant extrarenal disease or prerenal/postrenal azotemia at the time of initial diagnosis were excluded from the study. Animals with CRF and another disease which could cause secondary immunosuppression (such as, hyperadrenocorticism, hypoadrenocorticism, diabetes mellitus, neoplasia, pyometra) were excluded as well.

The group comprised 24 females (5 neutered) and 21 males (1 neutered) of various breeds. Average age was 9.85 y (from 5 mo to 16 y).

Dogs were divided into 3 groups according to the extent of clinical signs at the time of diagnosis. Dogs without clinical signs of CRF were placed in the 1st group. Azotemia was found by routine serum biochemistry examination and a diagnosis of chronic renal failure was confirmed by other findings (urinanalysis, ultrasonographic examination). The 2nd group included dogs with clinical signs of uremia (vomiting, inapetence, anorexia) and which responded well to therapy. The 3rd group included dogs that were in the end-stage of renal failure; these animals were not able to deal with changes in homeostasis and died or were euthanized shortly after diagnosis because of a lack of response to therapy. The characteristics of each group are listed in Table I.

Forty-five dogs were diagnosed with CRF according to history, clinical findings, haematological, and biochemical examination and urinanalysis. The diagnosis was confirmed by ultrasonography and in some cases by histopathological examination of renal tissue. The more severe clinical signs occurred when levels of creatinine and urea increased. In addition, these signs strongly correlated with diminishing numbers of red blood cells. Anemia observed in these animals was typically nonregenerative. The main biochemical parameters (serum creatinine, urea, and phosphorus) and red blood cells are shown in Table II. A typical finding was isostenuria and we found both active and passive urine sediment. In ultrasonography, the kidneys were mostly hyperechogenic. Diffuse chronic membranous glomerulonephritis was the most frequent observation in histopathology.

**Control dogs**

Fifteen clinically normal dogs (11 females (4 neutered); 4 males (1 neutered)) of various breeds that came to our clinic for vaccinations were included in the control group. Of similar age to the CRF dogs (average age 9.6 y), the control dogs were considered to be healthy based on their history, physical examination findings, and normal results of hematological and serum biochemistry analyses.

**Blood samples**

Where possible, blood samples were taken prior to any treatment. Some dogs were presented as acute patients, so they received fluids and other therapy (antiemetics, antulcerotic drugs) before

### Table I. Characteristics of dogs, number in each group, average age [mean ± standard deviation (s)] and their clinical signs

<table>
<thead>
<tr>
<th></th>
<th>Control dogs</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Subgroup 3 (lymphopenic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>15</td>
<td>10</td>
<td>15</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>11/4</td>
<td>7/3</td>
<td>6/9</td>
<td>8/12</td>
<td>5/5</td>
</tr>
<tr>
<td>Average age (y)</td>
<td>9.6 ± 2.9</td>
<td>11.4 ± 2.5</td>
<td>9.2 ± 4.3</td>
<td>9.4 ± 4.3</td>
<td>9.9 ± 3.6</td>
</tr>
<tr>
<td>Clinical signs</td>
<td>no</td>
<td>no</td>
<td>mild</td>
<td>severe</td>
<td>end-stage CRF</td>
</tr>
<tr>
<td></td>
<td>without azotemia</td>
<td>with azotemia</td>
<td></td>
<td>severe</td>
<td>end-stage lymphopenia</td>
</tr>
</tbody>
</table>

### Table II. Characteristics of dogs [creatinine, urea, and phosphorus levels, red blood (cell) counts expressed as a mean ± standard deviation (s)]

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Control dogs</th>
<th>Group 1</th>
<th>Group 2</th>
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<th>Subgroup 3 (lymphopenic)</th>
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</tr>
<tr>
<td></td>
<td></td>
<td>without azotemia</td>
<td>with azotemia</td>
<td></td>
<td>severe</td>
<td>end-stage lymphopenia</td>
</tr>
<tr>
<td>Creatinine</td>
<td>μmol/L</td>
<td>92.5 ± 16.9</td>
<td>194.8 ± 39.3</td>
<td>334.2 ± 136.4</td>
<td>939.8 ± 604.4</td>
<td>972.3 ± 658.2</td>
</tr>
<tr>
<td>Urea</td>
<td>mmol/L</td>
<td>6.7 ± 2.4</td>
<td>20.0 ± 8.3</td>
<td>28.0 ± 13.8</td>
<td>61.6 ± 34.2</td>
<td>62.7 ± 36.5</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>mmol/L</td>
<td>1.4 ± 0.5</td>
<td>1.8 ± 0.5</td>
<td>2.5 ± 1.2</td>
<td>5.6 ± 1.8</td>
<td>5.0 ± 1.8</td>
</tr>
<tr>
<td>Red blood cells</td>
<td>10(^12)/L</td>
<td>6.9 ± 0.9</td>
<td>6.0 ± 1.6</td>
<td>5.6 ± 1.3</td>
<td>4.8 ± 1.8</td>
<td>5.59 ± 1.9</td>
</tr>
</tbody>
</table>
blood samples were taken. The blood samples in these dogs were taken immediately. The dogs that had received corticosteroids were excluded from this study. The blood samples were taken by venipuncture from v. jugularis or v. cephalica antebrachii. The blood samples were collected into different tubes: EDTA tubes for hematological examination, standard biochemistry tubes and heparinised tubes for immunological examination. All examinations were carried out within 24 hours after collection.

**Immunological tests**

**Total and differential leukocyte counts**

Total leukocyte counts were determined using the Digicell 500 cell counter (Contraves AG, Zurich, Switzerland); differential leukocyte counts were enumerated from blood smears stained with May-Grunwald, Giemsa-Romanovski. Lymphocyte transformation test

The proliferation activity of lymphocytes was determined using the mitogen-driven lymphocyte transformation test (LTT). Mononuclear cells were isolated on a cell separating medium of density 1.077 (Histopaque 1077; Sigma-Aldrich Chemie, Munich, Germany). The density of the cell suspension was adjusted to 10^6/mL with RPMI 1640 medium with 10% of fetal calf serum. Twenty μL of mitogens — concanavalin A (ConA, 10 μg/mL; Pharmacia Biotech, AB, Sweden), pokeweed mitogen (PWM, 10 μg/mL; Sigma-Aldrich Chemie) were pipetted in triplicates into wells of microtiter plates. Antibodies CD3 (Ca17.2A12-IgG1), CD4 (CA13.1E14-IgG1), CD8 (CA9.JD3-IgG2a), CD21 (CA2.1D6-IgG1), γδ TCR (CA20.8H1-IgG2a), CD45 (CA12.10C12-IgG1), CD45RA (CA4.1D3-IgG1) provided by P.F. Moore and secondary goat anti mouse IgG1-FITC or IgG2a-PE antibodies (SouthernBiotech, Birmingham, Alabama, USA) were used. Propidium iodide was used to stain the DNA of dead and damaged cells and to exclude such events from analysis. Data were acquired on a standard FACS Calibur 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.

**Lymphocyte transformation test**

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a liquid scintillation counter (Top Count NXT; Packard Bioscience Instrument Company, Meriden, Connecticut, USA). The results were expressed as counts per minute (CPM) in stimulated samples versus CPM in nonstimulated controls.

Data analysis
Statistics were calculated with MS-Excel 6.0 [mean ± standard deviation (s)] and Graph Pad prism software (inter-group differences). Statistical differences between groups were estimated with the unpaired nonparametric Mann-Whitney test. Differences with $P < 0.05$ and $P < 0.01$ were interpreted as significant and highly significant, respectively. Correlations between parameters were calculated according the Spearman test.

## Results

### Total and differential leukocyte counts
Total and differential leukocyte counts are summarized in Table III. We found statistically important lymphopenia and observed decreases in the number of lymphocytes with increasing clinical signs and azotemia.

### Lymphocyte subsets
Corresponding to the decreased count of lymphocytes in groups of dogs with clinical signs, the counts of all main subsets also decreased. Nevertheless, a more considerable reduction was detected in CD21+ (B-cells), CD3^+CD4^-CD8^- (Tc-cells), and CD3^+CD4^- (NK subset). Lymphocyte subsets are presented in Table IV. A nonproportional decreased count of lymphocyte subsets was demonstrated by a statistically significant increase Th/Tc ratio (Figure 1). The T/B ratio tended to increase but not in statistically significant manner.

### Activity of lymphocytes
A significantly lower response to mitogens was observed in the group with severe clinical signs, especially in the lymphopenic dogs. The number of animals with depressed lymphocyte activity increased with the extent of clinical signs. We found a statistically very significant depression of response to pokeweed mitogen stimulation (Figure 2) and a statistically significant depression of response to concanavalin stimulation (data not shown).

### Correlation between parameters
There was a statistically significant negative correlation between the number of lymphocytes and the number of erythrocytes ($r = -0.3007, P = 0.0047, *$). There was a statistically significant positive correlation between the number of lymphocytes and their activity after stimulation with concanavalin A ($r = 0.4313, P = 0.0031, **$) and a very significant positive correlation between the number of lymphocytes and their activity after stimulation with pokeweed mitogen ($r = 0.5360, P = 0.0003, ***$).

### Discussion
Various abnormalities of the immune system have been demonstrated in humans with end-stage renal failure (2). Impaired polymorphonuclear functions, lymphopenia, and a lower response of lymphocytes to mitogens are well-described phenomena in such patients. Both cellular and humoral immunity are impaired (14,15). The clinical expression includes a high susceptibility to bacterial infection, prolonged allograft survival, cutaneous anergy, abnormal antibody responses to T-dependent antigens and to viral infection, and an increased incidence of *Mycobacterium tuberculosis* infection (5,16). Bacterial infection and sepsis are important causes of morbidity and mortality in people in the end-stage of renal disease (3).

The mechanisms of immunosuppression in uremia are only partially understood. Metabolic and toxic consequences of CRF and/or the compounding effects of malnutrition, vitamin deficiency, and drug therapy may each alone, or in any combination, contribute to the genesis of the deranged immune system in CRF. In most cases, the determination of the underlying cause of chronic renal failure is not possible. At the time of diagnosis, the organism is usually influenced by uremia per se, not by the primary disease.

Chronic renal failure is a very common disease especially in aging dogs and cats. Polyuria and polydipsia are usually the first clinical signs and are a consequence of a disturbed concentrating
ability of the renal tissue. Patients with polyuria are at risk of water-soluble vitamin deficiency (especially vitamin B and vitamin C). The excessive losses of these vitamins may contribute to poor immune responses in dogs with CRF. A deficiency in vitamins B6 and B12 adversely affects the cytotoxic activity of Tc-lymphocytes, decreases the number of T-cells (B12) and hampers the response of lymphocytes to mitogen (B6). Vitamin C deficiency leads to an increased susceptibility to bacterial or viral infections due to altered chemotaxis, a bactericidal activity of polymorphonuclears. It also lowers the lymphocyte response to mitogens (17).

Our results did not confirm this speculation, because all patients with CRF had polyuria (regardless of extent of clinical signs or serum creatinine levels) and we didn’t find any statistically important evidence of immunosuppression when all CRF dogs were compared with healthy controls. In addition, polyuria occurs before azotemia — in the phase of renal insufficiency (when 2/3 of the renal mass is lost).

A typical finding in patients with CRF is lymphopenia, both in humans and in dogs. Our study confirmed lymphopenia in dogs associated with CRF. The lymphopenia was more pronounced in dogs with the most severe clinical signs. Studies in uremic humans have shown that peripheral blood lymphocytes undergo accelerated apoptosis when cultured in vitro (6). Accelerated apoptosis may result from a higher expression of Fas (CD95) or a lower expression of Bcl-2 (6,7). Fas (CD95) is a widely expressed 45 KD membrane protein member of the tumor necrosis factor of cell surface molecules. The Fas molecule mediates apoptosis of T-lymphocytes following interaction with its natural ligand FasL (18). An increased expression of the Fas molecule has frequently been observed in uremic lymphocytes. Bcl-2 is a member of the Bcl-2 family with antiapoptogenetic 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 (19,20). CD69 antigen is a phosphorylated cell surface protein and a member of the C-type lectin family. Meier et al (7) described elevated levels of the CD69 antigen in chronically hemodialyzed patients. The induction of CD69 is an early biochemical event preceding T-cell proliferation in subjects with normal kidney function. But it is generally agreed that chronic hemodialysis patients show low T-cell proliferative activity (21). It has been discovered that levels of CD69 were significantly higher even in nondialyzed patients with CRF than in controls and a significantly high percentage of T-cells ultimately do not proliferate but become apoptotic (7).

Finally, apoptosis has been shown to be one of the mechanisms responsible for lymphopenia associated with aging. Because of the fact that CRF is very common in aging dogs and cats, all the patients and controls employed in the present study were age matched (22).

These observations suggest that the reduction in the numbers of T-cells in uremic patients may be due to a heightened susceptibility to apoptosis. Potential factors that may contribute to increased apoptosis include uremic toxins and reactive oxygen species; however, the exact mechanisms are still unclear. Lymphopenia per se does not explain the immune dysfunction described in uremic patients. The cytokine profile produced by T lymphocytes determines the immune response.

A decrease in lymphocytes was found in all main subsets in our study, but it was more evident in B- and Tc-cells; so the T/B and Th/Tc ratio increased with the extent of clinical signs. The depression in total T-cell counts (CD3+) is, in most studies, proportionally distributed between the Th (CD4+) and Tc (CD8+) and leads to no significant change in the Th/Tc ratio. A small number of studies have described a decrease in this ratio (23,24).

A significantly lower response to mitogens (concanaavalin A, pokeweed mitogen) was found in the group with the most severe clinical signs, especially in lymphopenic dogs. Peripheral blood mononuclear cells obtained from humans with chronic renal failure display depressed proliferative responses to phytohemagglutinin and concanaavalin A (25,26). Abnormal PHA induced proliferation does not necessarily signify T-cell abnormalities, since stimulation of proliferation by PHA requires the action of intact accessory cells (monocyte/macrophage). Proliferative hyporesponsiveness may be largely down to monocyte defects. Phytohemagglutinin and concanaavalin A are T-cell mitogens, whereas pokeweed mitogen acts on both T- and B-cells. The most significant decrease in lymphocyte response occurred after stimulation by pokeweed mitogen. Thus, the observed decrease in lymphocyte response may be associated with a more considerable fall in numbers of B-cells than of T-cells.

Normochromic, normocytic anemia is the most frequently noted hematological change in patients with chronic renal failure. The origin of this anemia is multifactorial and is not only caused by a lack of erythropoietin. Decreased erythrocyte lifespan, uremic inhibitors of erythropoiesis (such as spermine, spermidine, and parathyroid hormone), and bone marrow fibrosis due to secondary hyperparathyroidism may contribute to renal failure anemia. The number of lymphocytes was in a negative correlation with the number of erythrocytes in our study, so some of these factors might be associated with the altered immune response in CRF patients and may contribute not only to anemia, but also to lymphopenia.

Chronic renal failure is often complicated by secondary renal hyperparathyroidism with elevated levels of parathyroid hormone (PTH). It has been postulated that PTH may affect the immune system by increasing levels of cytosolic calcium and thereby stimulating T lymphocytes (27). High concentrations of intracellular calcium are responsible for many cellular dysfunctions. Previous data obtained from the incubation of normal lymphocytes with PTH were controversial. Human lymphocytes incubated with increasing amounts of PTH showed a considerable decrease in lymphocyte transformation and significant decrease in helpers to suppressors ratio (28). The T-cell proliferative response to PHA stimulation was significantly higher in lymphocyte cultures obtained from rats with high PTH levels than from normal rats (29). The proliferation of lymphocytes from uremic people incubated with PTH was significantly decreased. It suggests that the uremic state changes the response of T-cells to PTH (30).

Our finding of lymphopenia in uremic dogs is consistent with results reported in uremic humans. The causes of lymphopenia are likely the same as in humans suffering from CRF. Although we lack corresponding data from dogs with CRF, our findings of immunosuppression suggest that bacterial infection in uremic patients should be considered as a severe complication. Our finding of a correlation between lymphopenia and depressed lymphocyte activity
might have an practical conclusion: a diagnosis of lymphopenia by a routine hematological test in CRF dogs could signal an altered immune response; however, complicated immunological tests are not provided in practice.

Acknowledgments

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References