Canine Lymphoproliferative Disease Characterized by Lymphocytosis: Immunophenotypic Markers of Prognosis

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**Background:** Canine lymphoproliferative disease often presents with lymphocytosis and is immunophenotypically diverse.

**Hypothesis:** Immunophenotype predicts prognosis in canine lymphoproliferative disorders involving circulating lymphocytosis.

**Animals:** Dogs that had peripheral blood evaluation performed by flow cytometry by the Clinical Immunology Service at Colorado State University between 2003 and 2005.

**Methods:** Outcome data regarding treatment and survival were sought on patients with lymphocytosis comprising a single lymphocyte subset. Ninety-six patients that met the inclusion criteria had sufficient follow-up information to be included in the study.

**Results:** Four main phenotypic classifications were found: CD8+ T-cell, CD21+ B-cell, CD4-8-5+ (aberrant T-cell phenotype), and CD34+ (undifferentiated progenitor). Expression of CD34 predicted poor outcome with median survival of 16 days ($P < .0001$) compared with other phenotypes. Within the CD8+ phenotype, dogs presenting with a lymphocytosis >30,000 lymphocytes/µL had significantly shorter median survival (131 days) than those presenting with <30,000 lymphocytes/µL (1098 days, $P < .0008$). Within the T-cell leukemias, there was no difference in outcome between dogs with CD4-8-5+ leukemia and dogs with the CD8+ T-cell phenotype nor was the loss of expression of the pan-leukocyte marker CD45 associated with decreased survival time. A CD21+ lymphocytosis composed of large cells was associated with shorter survival time (129 days) than those with smaller circulating cells (median survival not reached, $P < .01$).

**Conclusions and Clinical Importance:** Immunophenotyping provides an objective method for determining prognosis in lymphoproliferative disorders characterized by lymphocytosis.

**Key words:** Flow cytometry; Immunophenotyping; Leukemia; Survival.

Canine lymphoproliferative disorders often present with peripheral lymphocytosis. Chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL), and lymphoma with circulating neoplastic cells (stage V lymphoma) have lymphocytosis as a primary feature. The distinction among these 3 diseases, however, is sometimes unclear. ALL was defined by a consensus group of clinical pathologists in 1991 as a disease in which ≥30% of the cells in the blood or marrow are lymphoblasts, and this consensus has been used since. However, acute leukemia can also involve lymph nodes and spleen leaving the distinction between stage V lymphoma and leukemia difficult to make. Similarly, although there is a general consensus that CLL is characterized by circulating small mature lymphocytes, this disease also may present with lymphadenopathy and splenomegaly, making the distinction between stage V small-cell lymphoma and CLL unclear. Furthermore, marrow involvement, which often has been used as a defining characteristic of leukemia in human medicine, is not a consistent feature of canine CLL.

Characterizing phenotypic markers in canine lymphoproliferative disorders could help to more accurately classify disease and predict clinical outcome. In this study, we carried out immunophenotyping of 96 cases of lymphoproliferative disorders involving peripheral lymphocytosis in dogs. Because of the lack of consensus definitions for distinguishing leukemia from stage V lymphoma, we did not attempt to distinguish between these entities. We found immunophenotype, together with 1 additional objective feature (lymphocyte size for B-cell disorders and initial lymphocyte count for T-cell disorders), to be highly prognostic. Our results suggest that immunophenotyping is a simple and objective method for deriving valuable prognostic information in patients with lymphoproliferative disorders involving peripheral blood.

**Materials and Methods**

**Study Subjects**

The study population included all dogs that had peripheral blood evaluation by flow cytometry in the Clinical Immunology Service at Colorado State University between 2003 and 2005. For the purpose of this study, we considered a process neoplastic if it met the following criteria: (1) A lymphocyte count above the reference range for the submitting laboratory or clinic; and one of the following: (2a) ≥80% of the lymphocytes had a single phenotype (eg, all B-cells); (2b) ≥60% of the lymphocytes had a single phenotype and a positive clonality assay (PARR assay or PCR for antigen receptor rearrangements); or (2c) expansion of lymphocytes with an aberrant phenotype for peripheral blood (eg, CD34+, loss of the pan-leukocyte antigen CD45, or expression of T-cell markers CD3 and CD5 but not the subset markers CD4 and CD8). Based on these criteria, 202 patients were enrolled in the study, and clinics were contacted for additional information: date of death or euthanasia or last follow-up visit, treatment data, CBC, and physical examination...
findings at submission. Survival time in days was calculated from the time of diagnosis or initial flow cytometry analysis to the time of death or euthanasia or last follow-up visit. Patients with splenomegaly or lymphadenopathy at presentation, documented by ultrasound examination, radiology, or physical examination, and with anemia or thrombocytopenia defined as an RBC or platelet count below reference interval at initial presentation, were further evaluated for survival time within each phenotypic subcategory. When no information about these features was provided, a normal value was not inferred and the patients were excluded from this secondary analysis. A detailed description of cellular morphology was not possible because freshly prepared blood films were not available for the majority of the samples.

Flow Cytometry

Samples were submitted to the Clinical Immunopathology Laboratory for flow cytometry at the discretion of the clinician. When given, reasons for submission included lymphocytosis on CBC, observation of an expanded population of small, mature-appearing cells, and identification of cells with an abnormal morphology by the clinical pathologist reviewing the blood smear. Peripheral blood in EDTA was shipped overnight with ice. The samples were processed on the day of arrival or the next day. Most samples were <72 hours old when processed but viability as determined by propidium iodide (PI) staining, rather than sample age, was used to determine if the sample was acceptable for flow cytometry. The percentage of PI-positive cells in each sample was determined before staining, and samples in which ≥50% of the cells were PI-positive were not stained or further analyzed in any way. The median percentage of dead cells in the CD34+ group was 13%, whereas in the other 3 groups the median was 7%. The true proportion of CD34+ cases may have been underestimated because a greater number of them were excluded because of a high proportion of dead cells.

Flow cytometric analysis was performed with 400 μL of whole blood. Red blood cells were lysed with 1 mL of lysis buffer (0.15 M NH₄Cl, 1 M KH₂PO₄, 0.1 m M Na₂ EDTA, 1 N HCl at a pH of 7.2–7.4) for 5 minutes at room temperature. Samples then were centrifuged and lysed a second time and resuspended in 200 μL of phosphate-buffered saline (PBS)-2% fetal bovine serum (FBS). A 96-well plate was used in which 25 μL of cell suspension was added to each of the 8 wells.

Directly conjugated antibodies were added to each well as follows: (1) no antibody, (2) CD45 phycoerythrin (PE) and isotype control (FITC) control, (3) CD18 FITC and isotype control PE, (4) CD8 FITC and CD4 PE, (5) CD5 FITC and B-cell PE, (6) CD45 PE and CD3 FITC, (7) CD4 FITC and CD14 PE, and (8) Class II FITC and CD34 PE. All samples were stained with the entire panel of antibodies. Samples were incubated for 15 minutes at room temperature and then washed twice. Samples then were resuspended in PBS-2%FBS with 10 μg/mL of PI for dead cell exclusion, and analyzed within 1/2 hour. The antibody clones (parentheses) were: anticanine CD3 (CA17.2A12), CD4 (YKIX302.9), CD5 (YKIX322.3), CD8 (YFCE118.3), CD45 (YKIX716.13), Class II MHC (YKIX334.2) and CD34 (1H6) and antihuMAN CD14 (UCHM1). Lymphocyte populations were gated on the basis of the forward scatter versus log side scatter plots after dead cells had been excluded by PI fluorescence. All cytometric analysis was performed with a Coulter XL flow cytometer, and data analysis was carried out with FCS Express.

Relative cell size was determined by measuring geometric mean linear forward scatter (FS). To discriminate between large and small cells a cut-off of 350 units was chosen. This value was chosen based on the typical size of lymphocytes in normal canine peripheral blood samples (data not shown). As an internal control, we also compared the forward scatter of the neoplastic cells with the forward scatter of the CD4+ CD14+ neutrophils. A ratio of ≥0.58 (FS lymphocytes/FS neutrophils) corresponded to large cells.

In this case series, all CD8+ and CD4+ CD8-5+ leukemias expressed cell surface CD3, indicating that these cells were not NK in origin.

PCR for Antigen Receptor Rearrangements

DNA from 200 μL of peripheral blood was isolated with a commercially available kit using previously described methods. The quantity of DNA added to each reaction was the equivalent of 6.7 μL whole blood and ranged between 100 and 500 ng. Amplification of immunoglobulin (Ig) and T-cell receptor γ sequences was performed using previously described primers and conditions. DNA positive controls were used to amplify the constant region gene of IgM on each sample along with negative controls to ensure no contamination was present. A positive reaction was defined as one or more prominent, discrete bands seen on gel electrophoresis. A negative reaction was defined as no clonal bands or a diffuse smear or ladder of faint bands representing a heterogeneous population of lymphocytes.

Statistical Analysis

Kaplan-Meier curves were used to evaluate the influence of phenotype and of thrombocytopenia, anemia, lymphadenopathy, splenomegaly, lymphocytosis, and lymphocyte count within each phenotype on survival. The log-rank test was used, with P < .05 indicating statistical significance. For each comparison, the Cox proportional hazards regression model was used to assess the death rate over time after diagnosis. Dogs lost to follow-up were censored in analyses as of the date of last follow-up visit. Fischer’s exact test was used to compare the incidence of clinical and hematological changes between phenotypic subsets, with P < .05 indicating statistical significance. The χ² test was used to compare breed, age, and sex distribution between phenotypes, with P < .05 indicating statistical significance.

Results

Patient Population

Two-hundred and two patients met the initial inclusion criteria. Upon initial analysis, 4 different major immunophenotypes (12 or more cases per group) were identified: CD21+ (B cell), CD8+ (T cell), CD8-5+ T cell (aberrant T cell phenotype), and CD34+ (unclassified as to lineage). Follow-up information was sought on these cases and sufficient medical history was obtained from 96 cases (34 B cell, 33 CD8+ T cell, 17 CD4-8-5+ T cell, and 12 CD34+ T cell). Survival time and treatment information was available on all 96 cases, whereas additional information (eg, organomegaly, anemia, thrombocytopenia) was available for only a subset of these cases. These cases were submitted from 47 clinics and 19 states representing all areas of the country. The median age of all 4 groups was similar (χ² P > .05). CD21 leukemias had a median age of 10.7 years with a range of 1–15 years; CD8 leukemias had a median age of 10 years with a range of 1–14 years, CD4–CD8–CD5+ leukemias had a median age of 9.7 years with a range of 2–14 years; and CD34 leukemias had a median age of 9 years with a range of 3–12 years. Sex was distributed equally within all groups (χ² P > .05).

Golden Retrievers had a significantly different distribution of immunophenotype compared with other
purebreds and all other dogs \( \chi^2 P = .01 \). There were no examples of B-cell lymphocytosis in Golden Retrievers, whereas 11 of the 13 cases were of T-cell origin. This finding is consistent with previous studies in which Golden Retrievers were over-represented in cases of T-cell lymphoproliferative disorders. \(^8\)

**Phenotypic Markers Predict Survival Time**

The median survival was compared among the phenotypic subsets. Expression of CD34 predicted a poor outcome with a median survival of 16 days (range, 3–128; \( P < .0001 \)) compared with other phenotypes (Fig 1). This was true despite the fact that 9/12 dogs with CD34+ circulating cells were treated with multidrug cyclophosphamide, doxorubicin, vincristine, and prednisone-based chemotherapy (CHOP). All of the samples analyzed were submitted based on a finding of lymphocytosis as described by the clinical laboratory performing the CBC, which suggests that these cells had the morphologic appearance of lymphocytes. These cells were consistently negative for all of the other lineage markers, however, and were not further subclassified as to phenotype.

When each of the remaining 3 immunophenotypic groups was examined as a whole, there was no significant difference in survival times among the phenotypes (Fig 1). The median survival of CD21 cases was 394 days, the median survival of CD4-8-5+ cases was 289 days, and that of CD8+ cases was 474 days. When considered in the context of additional data (presenting lymphocyte count and cell size), however, immunophenotype became highly prognostic.

**Presenting Lymphocyte Count Predicts Prognosis in CD8 T-Cell Leukemias**

Dogs with CD8+ lymphocytosis were categorized based on their initial presenting lymphocyte counts. All dogs with CD8+ lymphocytosis were subdivided into 4 quartiles based on initial lymphocyte counts. Survival experience based on Kaplan-Meier survival curves was similar in quartiles 1 and 2 as well as in quartiles 3 and 4, and consequently the groups were combined to form a lower and an upper half with 30,000 lymphocytes/µL as the cutoff value.

Dogs presenting with a CD8+ lymphocytosis of > 30,000 lymphocytes/µL had a significantly shorter median survival (131 days) than those presenting with <30,000 lymphocytes/µL (1,098 days, \( P = .0008 \); Fig 2). Based on Cox’s proportional hazards regression, this difference remained statistically significant after adjusting for therapy \( P = .037 \). Therapy in dogs with CD8+ lymphocytosis and an initial lymphocyte count of <30,000/µL consisted of a multidrug CHOP-based therapy in 9% of the patients, prednisone and chlorambucil in 18%, and prednisone alone in 18% of the cases. No treatment was administered in 55% of the patients. Therapy for dogs with a CD8+ lymphocytosis with an initial lymphocyte count >30,000/µL consisted of prednisone alone in 46% of the patients, prednisone and chlorambucil in 23%, and multidrug CHOP-based therapy in 23%, whereas 8% received no treatment.

All dogs with presenting lymphocyte counts <30,000 met our inclusion criteria for leukemia, but because *Ehrlichia canis* infection can cause both a preferential expansion of CD8 T cells and a clonal T-cell response, we sought results of *E. canis* serology on these cases. Eight of the 13 had negative serology, and information was unavailable for the remaining 5. Three of the remaining 5 cases were CD45+ T-cell lymphoma, supporting the diagnosis of a malignant disorder. One of the remaining 2 cases had a clonal T-cell population and lymphocyte counts up to 120,000 cells/µL at the time of euthanasia (2 years after diagnosis), making a reactive process unlikely. The final case for which there was no *E. canis* serology was treated with doxycycline, but the
lymphocytosis persisted for over a year after the initial diagnosis and treatment.

Cell Size Predicts Outcome in B-Cell Lymphoproliferative Disorders

CD21+ lymphocytosis composed of predominantly large cells was associated with shorter survival times (129 days) than CD21+ lymphocytosis composed of smaller cells; Kaplan-Meier survivorship function never decreased below 50% and, hence, median survival time could not be estimated (P = .01, Fig 3). Again, using Cox’s proportional hazards, this finding was shown to be independent of therapy (P = .035). Patients with large cell CD21+ lymphocytosis were treated with multidrug therapy in 87% of the cases and prednisone alone in 13%. Patients with small cell CD21+ lymphocytosis were treated with multidrug therapy in 78% of the cases, prednisone and chlorambucil in 11%, and prednisone alone in 11% of the cases. Presenting lymphocyte count did not significantly influence survival in dogs with CD21+ lymphocytosis, and, in fact, the trend for survival was the opposite of that seen with CD8+ cases. Dogs presenting with >30,000 CD21+ lymphocytes/µL had a median survival of 666 days whereas those presenting with <30,000 CD21+ lymphocytes/µL had a median survival of 394 days (P = .12).

In all cases of CD21+ lymphocytosis involving small cells and therefore a good prognosis, >80% of the lymphocytes were CD21+. More than 60% of the animals had absolute lymphocyte counts >30,000/µL, and the lowest absolute CD21+ count was 6318 cells/µL, which is a 21-fold increase above our reference range. Because there are no known non-neoplastic processes in dogs that result in a persistent, homogeneous expansion of B cells, we considered all these cases to be neoplastic.

Aberrant Surface Marker Expression

Seventeen cases of lymphocytosis in which the cells expressed both T-cell markers CD3 and CD5, but were negative for both subset markers CD4 or CD8, were evaluated. This aberrant phenotype was not associated with a worse prognosis when compared with all CD8 T-cell leukemias (CD4-8-5+ cases, median survival 289 days; CD8+ cases, median survival 474 days, P = .72); only two of these cases had initial lymphocyte counts >30,000/µL, and we could not determine if presenting lymphocyte count is prognostically useful in this subset of T-cell disorders.

Twelve cases of T-cell lymphocytosis (in both CD8+ and CD4-8-5+ categories) were negative for the panleukocyte antigen CD45, a feature that was best identified with the combination of CD45-PE and CD3-F. This phenotype was not significantly associated with a worse outcome (CD45+ cases, median survival 397 days; CD45− cases, median survival 285 days, P = .52)

Other Clinical and Hematological Variables

Information about the presence of lymphadenopathy, splenomegaly, thrombocytopenia, and anemia was available for a proportion of the patients. Table 1 shows how each of these abnormalities was distributed among the phenotypic subgroups. We detected significant differences in the presence of lymphadenopathy and

<table>
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<th>CD8</th>
<th>Total cases</th>
<th>Anemia</th>
<th>Thrombocytopenia</th>
<th>Lymphadenopathy</th>
<th>Splenomegaly</th>
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<td>&gt;30,000</td>
<td>19</td>
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<td>6/13 (46%)</td>
<td>7/11 (63%)</td>
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<tr>
<td>&lt;30,000</td>
<td>14</td>
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<td>1/10 (10%)</td>
<td>5/11 (45%)</td>
<td>0/7 (0%)</td>
</tr>
<tr>
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<td>Large</td>
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<td>9/13 (69%)</td>
<td>8/11 (72%)</td>
<td>13/13 (100%)</td>
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<tr>
<td>Small</td>
<td>21</td>
<td>8/21 (38%)</td>
<td>6/21 (29%)</td>
<td>8/20 (40%)</td>
<td>4/14 (29%)</td>
</tr>
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*Number of cases with the reported finding/number of cases for which data were available.

bPercentage of cases with the reported finding.
thrombocytopenia between the small and large B-cell groups. All of the dogs with large B cells had lymphadenopathy, whereas half of those with small B cells had lymphadenopathy (Fischer’s exact test, \( P = .005 \)). Similarly, the large B-cell group was significantly more likely to have thrombocytopenia than was the small B-cell group (Fischer’s exact test, \( P = .027 \)). Regardless of presenting lymphocyte count (CD8 lymphocytosis) or lymphocyte size (CD21 lymphocytosis), lymphadenopathy was present in \( \geq 40\% \) of all patients in all groups, emphasizing the difficulty in classifying disorders in which both lymphocytosis and lymphadenopathy are present.

Insufficient cases were available to allow us to examine the effect of each of the above variables on survival within subcategories (high and low CD8 lymphocyte count, large and small CD21+ cells). However, when examined in the combined groups (all CD8+ and all CD21+ cases), some of the above clinical variables had significant impact on survival. Both lymphadenopathy and platelet count influenced survival in the CD21 group. Dogs with lymphadenopathy had a median survival of 173 days, and those without lymphadenopathy had a median survival of 666 days (\( P = .004 \)). Dogs with thrombocytopenia had a median survival of 133 days, whereas those with normal platelet counts survived significantly longer (undefined, \( P = .023 \)). Lymphadenopathy and thrombocytopenia were not significantly associated with outcome in the CD8 group. Neither splenomegaly nor anemia was significantly associated with survival in either group.

**Discussion**

The results of this retrospective study indicate that the immunophenotype of neoplastic canine lymphocytosis provides important prognostic information. Because no consensus on the distinction between leukemia and the leukemic phase of lymphoma exists in the veterinary literature, and because some forms of leukemia are considered to be the same disease as lymphoma in human patients (eg, CLL and small lymphocytic lymphoma\(^4\)), we designed this study to examine the relationship between immunophenotype and prognosis independently of clinical diagnosis.

Expression of CD34 predicted poor outcome with a median survival of 16 days. Our findings support the suggestion of Vernau et al\(^{4,12}\) that CD34 is a marker of acute leukemia. Morphologic description alone can be insufficient to differentiate chronic from acute lymphocytic leukemia\(^4\) and to distinguish subclassifications of acute leukemias. Therefore, the availability of a simple objective test for identifying this group of diseases may be a useful tool for clinicians. Because CD34+ cells do not stain with any of the other surface markers used in our laboratory, future studies aimed at differentiating cells of myeloid and lymphoid origin may aid in evaluating improved treatment strategies targeted at different CD34+ sublineages.

Although there was no overall difference in survival between dogs with T-cell and B-cell lymphocytosis, additional features in each of these groups were significantly associated with survival. For CD8+ T-cell disease, the absolute lymphocyte count at presentation predicted survival. The CD8+ dogs in our study presenting with lymphocytosis > 30,000 lymphocytes/μL had significantly shorter median survival (median survival = 131 days) than those presenting with < 30,000 lymphocytes/μL (median survival = 1,098 days). The difference remained significant after taking treatment into account.

A question raised by our data is whether or not low lymphocyte count and high lymphocyte count CD8+ T-cell disorders are the same disease process identified at early and late stages, respectively, or whether they represent distinct neoplastic processes. Prospective, longitudinal studies will be necessary to answer this question.

The overall size of the circulating lymphocytes was found to predict outcome in cases of CD21+ lymphocytosis. CD21+ lymphocytosis composed of large cells was associated with significantly shorter survival time (median survival = 129 days) than those with small circulating cells (median survival time not reached). Because we included all dogs with lymphocytosis regardless of lymphocyte morphology or peripheral lymphadenopathy, it is likely that we included cases of both primary leukemia and stage V lymphoma. Given that the majority of canine multicentric lymphomas are of B-cell phenotype\(^,4,13\) and that all cases involving large circulating B cells had lymphadenopathy, we suggest that cases of large cell lymphocytosis most likely represented stage V B-cell lymphoma. We cannot exclude, however, the possibility that some cases involving large B cells might have been the result of CLL transformation or blast crisis. Richter’s Syndrome (RS) describes the transition from human B-cell CLL/small lymphocytic lymphoma to high-grade non-Hodgkin’s lymphoma. The cells in RS are larger and are associated with a poor response to therapy and rapid disease progression.\(^16\)

There was no significant difference in overall survival times when cases of CD45– lymphocytosis were compared with those with preserved CD45 surface staining. This is in contrast to some human lymphoproliferative disorders in which loss of CD45 expression is associated with both shorter and longer survival times. Although aberrant surface molecule expression does not appear to correlate with survival in dogs, it is a useful feature to confirm neoplastic transformation. Loss of CD45 and other T-cell markers is considered diagnostic for malignancy in human patients.\(^10\)

Our findings indicate considerable prognostic value in determining immunophenotype in dogs with circulating neoplastic lymphocytes. Although the overall survival of dogs with T and B lymphoproliferative disorders with peripheral blood involvement is not different, classifying these disorders by phenotype allows us to evaluate other features that are associated with prognosis. One of the advantages of using flow cytometry for classifying lymphoproliferative disorders is that this approach is more objective than cytology or histology, and may be easier to standardize. Additional studies should be directed at correlating cytologic and histologic findings...
with survival and immunophenotypic properties, with the goal of developing common criteria for classifying canine lymphoproliferative disorders using commercially available reagents.

Footnotes

a Serotec Inc, Raleigh, NC
b B-D Biosciences, San Jose, CA
c Beckman Coulter Inc, Fullerton, CA
d De Novo Software, Ontario, Canada

Acknowledgments

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References