Flow cytometric evaluation of peripheral blood and bone marrow and fine-needle aspirate samples from multiple sites in dogs with multicentric lymphoma

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Objective—To determine whether the extent of disease in dogs with lymphoma can be assessed via flow cytometry and to evaluate the suitability of fine-needle aspirates from the liver and spleen of dogs for flow cytometric examination.

Animals—44 dogs with multicentric B-cell (n = 35) or T-cell lymphoma (9) and 5 healthy control dogs.

Procedures—Peripheral blood and bone marrow samples and fine-needle aspirates of lymph node, liver, and spleen were examined via flow cytometry. Logarithmically transformed T-cell–to–B-cell percentage ratio (log(T:B)) values were calculated. Thresholds defined by use of log(T:B) values of samples from control dogs were used to determine extranodal lymphoma involvement in lymphoma-affected dogs; results were compared with cytologic findings.

Results—12 of 245 (5%) samples (9 liver, 1 spleen, and 2 bone marrow) had insufficient cellularity for flow cytometric evaluation. Mean log(T:B) values of samples from dogs with B-cell lymphoma were significantly lower than those of samples from the same site in dogs with T-cell lymphoma and in control dogs. In dogs with T-cell lymphoma, the log(T:B) of lymph node, bone marrow, and spleen samples was significantly higher than in control dogs. Of 165 samples assessed for extranodal lymphoma involvement, 116 (70%) tested positive via flow cytometric analysis; results agreed with cytologic findings in 133 of 161 (83%) samples evaluated via both methods.

Conclusions and Clinical Relevance—Results suggested that flow cytometry may aid in detection of extranodal lymphoma involvement in dogs, but further research is needed. Most fine-needle aspirates of liver and spleen were suitable for flow cytometric evaluation.

Flow cytometry is a technique that allows assessment of large numbers of cells with simultaneous recording of different phenotypic variables for each individual cell. Flow cytometry has become an established tool for immunophenotype determination in dogs with lymphoma. For this purpose, enlarged peripheral lymph nodes of dogs affected by multicentric lymphoma have been investigated in many flow cytometry-based studies. Blood and bone marrow from dogs with lymphoma have been studied with this technique as well. To the authors’ knowledge, comparable studies to evaluate lymphocytes obtained in fine-needle aspirate samples from the liver or spleen of lymphoma-affected dogs have not been reported. Investigators of previous studies used flow cytometry to evaluate tissue samples obtained from the liver or spleen of dogs via biopsy or necropsy. The objective of the study reported here was to determine whether the extent of disease in dogs with multicentric lymphoma can be assessed via flow cytometry. We also sought to evaluate the suitability of fine-needle aspirates from the liver and spleen of dogs for use in flow cytometric examination.

Materials and Methods

Dogs—Dogs with cytologically confirmed, multicentric lymphoma newly diagnosed between August 1, 2007, and August 31, 2009, were eligible for enrollment in the study. A diagnosis of B-cell or T-cell lymphoma confirmed via flow cytometric evaluation of lymph node fine-needle aspirate samples with antibodies against CD21 or CD3, respectively, was required for inclusion. Dogs that had received chemotherapy prior to the study were excluded, whereas those treated with glucocorticoids were not. Written owner consent was obtained for all client-owned dogs prior to study enrollment. Five Beagles owned by University of Veterinary Medicine Hannover were included as controls; these dogs were determined to be healthy on the basis of results of physical examination, CBC, serum biochemical analysis, thoracic and abdominal radiography, and abdominal ultrasonography. The study design was reviewed and approved by the governmental animal care committee.

Staging—Clinical staging of dogs with lymphoma was performed according to the World Health Organization clinical staging system. Dogs were evaluated on the basis of results of physical examination, CBC, serum biochemical analysis, thoracic and abdominal radiography, and cytologic evaluation of a bone marrow aspirate.

Sample collection—Blood and bone marrow samples were collected in EDTA-containing tubes. Blood (at least 1 mL) was collected from a cephalic or saphenous vein, and bone marrow was aspirated from an iliac crest with an 18-gauge spinal needle. Fine-needle aspirates from palpable lymph nodes were obtained with a 21-gauge needle. With ultrasonographic guidance, 22-gauge needles were used to obtain fine-needle aspirate samples from the liver and spleen. Two samples were collected from each of these organs; one was used to prepare smears for cytologic evaluation, and the other was suspended in 1 mL of RPMI-1640 medium supplemented with 10% fetal calf serum in an EDTA-containing tube for flow cytometric analysis. All samples were stored at approximately 4°C until processed ≤12 hours after collection. When anesthesia was required for sample collection, propofol was administered IV and titrated to effect at the attending clinician’s discretion.

Flow cytometry sample preparation—Cell counts in all samples were measured with an automated blood cell counter. Samples were diluted in PBS solution to a maximum concentration of 10,000 nucleated cells/µL if the concentration exceeded this value. Sample aliquots (100 µL) were incubated with 10 µL of directly conjugated monoclonal antibodies against canine cell surface antigens for 15 minutes at room temperature (approx 20°C) in the dark; the antibodies used included a far-red fluorescent dye–conjugated rat anti-canine CD45 antibody (YKIX716.13), fluorescein isothiocyanate-conjugated mouse anti-canine CD3 antibody (CA17.2A12), and R-phycoerythrin-conjugated mouse anti-canine CD21 antibody (CA2-1D6). Isotype-matched control antibodies (far-red fluorescent dye–conjugated rat IgG2b [LO-DNP-11], fluorescein isothiocyanate-conjugated mouse IgG1 [DAK-GO1], and R-phycoerythrin-conjugated mouse IgG1 [WS2/25]) were used for negative control staining. Following incubation with anti-canine or isotype control antibodies, RBC lysis was performed. Two milliliters of erythrocyte lysing solution, diluted according to the manufacturer’s instructions, was added to each tube, and the sample was incubated for another 15 minutes at room temperature in the dark. Subsequently, cells were washed twice in PBS solution, resuspended in sheath fluid, and analyzed immediately.

Flow cytometric analyses—Analysis was performed with a multicolor flow cytometer, which was calibrated once daily when in use. Where possible, data for ≥10,000 events/tube (ie, 10,000 live cells/tube) were analyzed. In tubes with low concentrations of cells, as many events as possible were acquired. In the first 9 blood samples and the first 8 bone marrow samples, 10,000 events were acquired. Because high percentages of nucleated erythrocyte precursors (which are resistant to lysis) were present, especially in bone marrow samples, and because of the species-specific predominance of granulocytes in canine blood, the event count in blood and bone marrow samples of the remaining patients enrolled in the present study was increased to 50,000 events. Other acquisition settings were kept constant throughout the study.

Flow cytometry data evaluation—The data were analyzed by use of flow cytometry data acquisition and analysis software. Data were visualized as dual-parameter correlated plots (dot plots).

Cells incubated with isotype control antibodies and cells that tested negative for the antigen of interest in samples incubated with anti-canine antibodies were used as negative controls. On the basis of FSC and SSC properties and staining for CD45, gates were set to prepare smears for cytologic examination, and the
<1,000 mononuclear leukocytes were excluded from further analyses. The percentages of CD3-positive, CD21-negative cells (T cells) and CD21-positive, CD3-negative cells (B cells) within the mononuclear leuko-
cytes were determined (Figure 2). The T:B values were calculated and logarithmically transformed (base 10). Mean values of the log(T:B) ± 2 SD in healthy control dogs were used to define upper and lower threshold values for each sample type. Samples from patients with T-cell lymphoma were considered positive for lymphoma involvement if the log(T:B) was greater than the upper threshold value. Samples from patients with B-cell lymphoma were considered positive for lymphoma involvement if the log(T:B) was less than the lower threshold value. To compare the relative size, granularity, and complexity of cells as well as expression of cell surface antigens, mean FSC, SSC, and fluorescence intensity (staining) values of cells were recorded. All flow cytometric analyses were performed by 1 investigator (AEJ).

Cytologic examination—Smears for cytologic examination were stained with Pappenheim stain and evaluated via light microscopy by an experienced certified clinical pathologist (RM). Extramedullary lymphoma involvement was diagnosed in cytologic specimens if a distinct lymphoma cell population could be defined; these samples included an estimated number of lymphoma cells that was > 1%, > 2%, or ≥ 5% of nucleated cells for samples obtained from peripheral blood, bone marrow, or spleen, respectively. Samples indicative of extramedullary lymphoma involvement of the liver contained ≥ 5 lymphoma cells/hpf (magnification, ×400).

Suspected extranodal lymphoma involvement was determined if percentages of suspected lymphoma cells in these samples were less than the described values or if a subjectively large number of blast cells of different lineages (eg, cells suggestive of extramedullary hematoepoiesis in the liver or increased granulopoietic activity in the bone marrow) resulted in a less definitive assessment. In the comparison of cytologic and flow cytometric data, samples from sites diagnosed as having lymphoma involvement and those with suspected lymphoma involvement were treated equally.

Statistical analysis—A Kolmogorov-Smirnov test was performed to verify the assumption of normality of T:B and log(T:B) values in lymph node, blood, bone marrow, liver, and spleen samples. The log(T:B) values were evaluated for significant differences between samples from dogs with T-cell and B-cell lymphoma and between samples from dogs with each type of lymphoma and healthy control dogs. If results of 1-way ANOVA indicated significant differences between sample groups, pairwise comparisons were performed by means of a 1-sided t test (for equal or unequal variances, as applicable) with Bonferroni correction for multiple tests. Values of P < 0.05 were considered to be significant (P < 0.0167 in the Bonferroni-adjusted t test). Statistical analysis was performed with commercially available software.

Results

Dogs—Forty-four dogs with lymphoma were included in the study. Breeds included mixed (n = 12), Rottweiler (3), Boxer (3), Small Munsterlander (2), Bernese Mountain Dog (2), English Cocker Spaniel (2), Dachshund (2), Golden Retriever (2), and Hovawart (2) and 1 each of Beagle, Bracco Italiano, Bullmastiff, German Shepherd Dog, Irish Terrier, Jagdterrier, Labrador Retriever, Malinois, Miniature Schnauzer, Norwich Terrier, American Pit Bull Terrier, and Shetland Sheepdog. Median age was 7.5 years (range, 2 to 16 years) and median body weight was 30.5 kg (range, 8.5 to 52.7 kg). There were 15 female (7 spayed) and 29 male (11 castrated) dogs. Lymphoma was classified as stage III in 4 (9%) dogs, stage IV in 17 (39%), and stage V in 23 (52%). Lymphoma was further classified as stage a in 21 (48%) dogs and stage b in 23 (52%).

Four of the 5 healthy Beagles were 7 years old, and 1 was 9 years old. Median body weight of these dogs was 18.7 kg (range, 16.2 to 19.6 kg). Two were spayed females, and 3 were castrated males.

Suitability of samples for flow cytometry—In the 44 dogs with lymphoma, ≥ 10,000 events/tube were acquired in all blood and bone marrow samples (n = 44 samples of each type) as well as in 43 (98%) lymph node samples, 29 (66%) liver samples, and 39 (89%) spleen samples. Eight liver samples, 2 bone marrow samples, and 1 spleen sample were excluded from further analyses because of insufficient cellularity (< 1,000 mononuclear leukocytes). Ten of the 11 excluded samples were analyzed within the first year of the study period.

In the 5 healthy control dogs, ≥ 10,000 events/tube were acquired in all lymph node, blood, and bone marrow samples and in 4 of 5 spleen samples. In all 5 liver samples, < 10,000 events/tube were acquired, and 1 of these samples was excluded from further analyses because of low cellularity.

In total, 233 of 245 (93%) samples were suitable for flow cytometric analysis. Regarding fine-needle aspirates of liver and spleen, 88 of 98 (90%) samples had sufficient cellularity for flow cytometry.

Table 1—Mean ± SD values of T:B and log(T:B) determined via flow cytometric examination of blood, bone marrow, liver, and spleen samples from 5 healthy control dogs and 44 dogs with B-cell (n = 35) or T-cell (9) lymphoma.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>No. of samples</th>
<th>T:B</th>
<th>log(T:B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph node</td>
<td>5</td>
<td>3.04 ± 1.28</td>
<td>0.45 ± 0.20</td>
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<tr>
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<td>7.46 ± 2.66</td>
<td>0.85 ± 0.15</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>5</td>
<td>3.39 ± 3.40</td>
<td>0.40 ± 0.49</td>
</tr>
<tr>
<td>Liver</td>
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<td>4.22 ± 10.82</td>
<td>1.08 ± 0.27</td>
</tr>
<tr>
<td>Spleen</td>
<td>5</td>
<td>2.27 ± 0.90</td>
<td>0.33 ± 0.18</td>
</tr>
<tr>
<td>B-cell lymphoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph node</td>
<td>35</td>
<td>0.15 ± 0.33</td>
<td>–1.11 ± 0.47</td>
</tr>
<tr>
<td>Blood</td>
<td>35</td>
<td>3.20 ± 3.97</td>
<td>0.12 ± 0.64</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>34</td>
<td>1.25 ± 1.35</td>
<td>–0.22 ± 0.59</td>
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<tr>
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<td>1.63 ± 2.67</td>
<td>–0.24 ± 0.61</td>
</tr>
<tr>
<td>Spleen</td>
<td>34</td>
<td>0.20 ± 0.21</td>
<td>–0.83 ± 0.44</td>
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<td>T-cell lymphoma</td>
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<td></td>
<td></td>
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<tr>
<td>Lymph node</td>
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<td>103.24 ± 163.68</td>
<td>1.60 ± 0.60</td>
</tr>
<tr>
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<td>1.21 ± 0.54</td>
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<td>Bone marrow</td>
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<td>18.49 ± 14.64</td>
<td>1.11 ± 0.43</td>
</tr>
<tr>
<td>Liver</td>
<td>7</td>
<td>104.44 ± 98.83</td>
<td>1.73 ± 0.63</td>
</tr>
<tr>
<td>Spleen</td>
<td>9</td>
<td>26.24 ± 58.87</td>
<td>1.17 ± 0.53</td>
</tr>
</tbody>
</table>

Twelve samples were excluded from analysis because of insufficient cellularity.

*Values of log(T:B) were significantly different, compared with the log(T:B) value for control samples. †Values of log(T:B) were significantly different between B-cell and T-cell lymphoma samples.
Median mononuclear leukocyte counts in fine-needle aspirates from lymphoma-affected dogs were 8,656 leukocytes (range, 5,715 to 9,604 leukocytes) for lymph node samples, 2,766 leukocytes (range, 60 to 8,949 leukocytes) for liver samples, and 6,336 (range, 559 to 9,265 leukocytes) for spleen samples; values for bone marrow and blood samples from lymphoma-affected dogs were 7,127 leukocytes (range, 497 to 32,025 leukocytes) and 9,526 leukocytes (range, 1,471 to 39,945 leukocytes), respectively. In healthy control dogs, median mononuclear leukocyte counts in fine-needle aspirates were 9,330 leukocytes (range, 9,089 to 9,557 leukocytes) for lymph node samples, 1,229 leukocytes (range, 396 to 1,364 leukocytes) for liver samples, and 5,656 leukocytes (range, 3,867 to 5,904 leukocytes) for spleen samples; values for bone marrow and blood samples in these dogs were 5,399 leukocytes

Figure 3—Box-and-whiskers plots depicting values of T:B on a logarithmic scale for control dogs (n = 5), dogs with B-cell lymphoma (35), and dogs with T-cell lymphoma (9) in lymph node (A), peripheral blood (B), bone marrow (C), liver (D), and spleen samples (E). Eight samples from dogs with B-cell lymphoma (6 liver, 1 bone marrow, and 1 spleen), 3 samples from dogs with T-cell lymphoma (2 liver and 1 bone marrow), and 1 liver sample from a healthy control dog were excluded from analysis because of insufficient cellularity. Upper and lower limits of the boxes represent the upper and lower quartiles, respectively. Solid lines within boxes represent the median value. Whiskers extend to the minimum and maximum sample values; outliers (values were > 1.5 times the interquartile range) are represented by solid circles. Significant (P < 0.0167, appropriate to an α of 0.05 with Bonferroni correction for 3 pairwise comparisons) differences in log(T:B) are indicated (asterisk).
Immunophenotype of lymphoma cells—On the basis of flow cytometric evaluation of cell-specific antigen expression in lymph node fine-needle aspirate samples, B-cell lymphoma was diagnosed in 35 of 44 (80%) affected dogs. Nine (20%) dogs had T-cell lymphoma.

Flow cytometric assessment of extranodal involvement—Results of Kolmogorov-Smirnov testing supported the assumption of normality of log(T:B) values for all samples, whereas T:B values were not normally distributed for some sample types (lymph node and liver samples from dogs with B-cell lymphoma). Results of ANOVA revealed that log(T:B) values were significantly (P < 0.05 for all comparisons) different among samples from dogs with B-cell lymphoma, dogs with T-cell lymphoma, and healthy control dogs. Evaluation of t test results revealed that mean log(T:B) values of lymph node, blood, bone marrow, liver, and spleen samples from dogs with B-cell lymphoma were significantly lower than those in samples of the same tissue type from dogs with T-cell lymphoma (P < 0.001 in all sample types) and in healthy control dogs (P < 0.001 in lymph node, blood, liver, and spleen samples; P = 0.017 in bone marrow). These values were summarized and evaluated graphically (Table 1; Figure 3). In dogs with T-cell lymphoma, mean log(T:B) values of lymph node (P = 0.002), bone marrow (P = 0.009), and spleen (P < 0.001) samples were significantly higher than those in healthy controls. The log(T:B) in blood (P = 0.046) and liver (P = 0.022) samples of dogs with T-cell lymphoma after Bonferroni correction was not significantly different from that of samples from healthy control dogs.

Defined thresholds for log(T:B) values were determined for blood (upper limit, 1.14; lower limit, 0.56), bone marrow (upper limit, 1.38; lower limit, −0.59), liver (upper limit, 1.62; lower limit, 0.54), and spleen (upper limit, 0.68; lower limit, −0.02) samples. On the basis of these thresholds, 116 of 165 (70%) evaluable samples collected from the blood, bone marrow, liver, and spleen of dogs with lymphoma were considered positive for lymphoma involvement. Among these, results were positive for 29 of 44 (66%) blood samples (25 from dogs with B-cell lymphoma and 4 from dogs with T-cell lymphoma) and 15 of 42 (36%) bone marrow samples (11 from dogs with B-cell lymphoma and 4 from dogs with T-cell lymphoma). Results were also positive for 30 of 36 (83%) liver samples (26 from dogs with B-cell lymphoma and 4 from dogs with T-cell lymphoma), and 42 of 43 (98%) spleen samples (34 and 8 from dogs with B-cell and T-cell lymphoma, respectively). By use of the log(T:B), 9 of 35 dogs with B-cell lymphoma and 2 of 9 dogs with T-cell lymphoma were considered positive for lymphoma involvement in blood, bone marrow, liver, and spleen samples, and 2 dogs with B-cell lymphoma that had liver samples excluded from analysis had positive results in all remaining samples.

In 30 of 35 (86%) dogs with B-cell lymphoma, a population of presumed neoplastic B cells was identified in ≥1 sample site via flow cytometry. These were mononuclear leukocytes that had higher mean FSC and SSC values, higher mean CD21 expression density, or both, as determined via subjective evaluation of dot plots, compared with presumed nonneoplastic B-cell population for which these values were within expected limits. The other 5 dogs with B-cell lymphoma had either almost complete depletion of apparently normal B cells in all sample sites or a confluence of presumed neoplastic and nonneoplastic B cells to 1 indistinguishable population (Figure 4). Of the 9 dogs with T-cell lymphoma, a separation of CD3-positive cells into 2 distinct populations was detected in only 1 dog: in liver and spleen samples from that dog, presumed neoplastic T cells had an apparently higher mean FSC value than did T cells that were considered nonneoplastic. In the lymph node sample of the same dog, all detected T cells had a subjectively high mean FSC value so that depletion of nonneoplastic T cells in that sample was assumed.

On the basis of subjectively assessed fluorescence intensity, relative CD21 expression density was higher in presumed neoplastic than in nonneoplastic lymphocytes in ≥1 sample from 24 of 33 (69%) dogs with B-cell lymphoma. A decrease in relative CD21 expression...
density was not observed in any presumed neoplastic B-cell populations. No difference in relative CD3 expression density was apparent between presumed neoplastic and nonneoplastic T-cell populations. The B cells in spleen samples of all 5 healthy control dogs segregated into 2 distinct populations on the basis of high or low relative CD21 expression density as assessed in dot plots. The population that had high CD21 expression density also had apparently higher mean FSC and SSC values, compared with the population that had low CD21 expression density (Figure 4). This segregation of B-cell populations was also detected in spleen samples from 6 of 9 dogs with T-cell lymphoma.

Cytologic examination for extranodal lymphoma involvement—Four samples were excluded from cytologic evaluation because of disruption of cells (1 blood sample), scant cellularity in the prepared smear (1 bone marrow sample), or excessive blood contamination of the smear (1 liver and 1 spleen sample). Of the remaining 172 samples, 111 (65%) were considered positive for extranodal lymphoma involvement and 21 (12%) had suspected extranodal lymphoma involvement.

Results were positive for 25 of 43 (58%) blood samples (22 from dogs with B-cell lymphoma and 3 from dogs with T-cell lymphoma) and 16 of 43 (37%) bone marrow samples (13 from dogs with B-cell lymphoma and 1 from a dog with T-cell lymphoma). Results were also positive for 30 of 43 (70%) liver samples (27 from dogs with B-cell lymphoma and 3 from dogs with T-cell lymphoma) and 40 of 43 (93%) spleen samples (35 and 3 from dogs with B-cell and T-cell lymphoma, respectively).

Lymphoma involvement was suspected in 10 bone marrow samples (8 from dogs with B-cell lymphoma and 2 from dogs with T-cell lymphoma), 9 liver samples (6 from dogs with B-cell lymphoma and 3 from dogs with T-cell lymphoma), 1 blood sample from a dog with B-cell lymphoma, and 1 spleen sample from a dog with T-cell lymphoma. In total, 161 of 176 (91%) samples were evaluated both cytologically and by means of flow cytometry, and positive and negative results for detection of lymphoma involvement were compared (Table 2). Results of cytologic and flow cytometric examination were in agreement for 133 (83%) samples and in disagreement for 28 (17%) samples. Among the 28 samples for which results were in disagreement, 11 had positive results for flow cytometric examination with negative results for cytologic evaluation, whereas the other 17 samples had negative results for flow cytometric examination and were deemed positive for lymphoma involvement (n = 8) or lymphoma involvement was suspected (9) via cytologic examination.

### Discussion

The objective of the study reported here was to determine whether the extent of disease in dogs with multicentric lymphoma can be assessed via flow cytometry and to evaluate the suitability of fine-needle aspirates from the liver and spleen of dogs for flow cytometric examination. To the authors’ knowledge, flow cytometric analyses of liver and spleen samples from dogs with lymphoma has not been previously reported.

Some flow cytometry-based studies have focused on lymphocyte development in lymphatic organs (including the spleen) or the characterization of lymphocytes found in the liver in healthy dogs. In an earlier study, investigators analyzed T-cell subsets in lymph nodes, spleen, bone marrow, thymus, and blood of healthy dogs by use of monoclonal antibodies. Another flow cytometry-based study investigated mononuclear cells in the spleen of dogs infected with *Leishmania infantum* (also called *Leishmania chagasi*).

Data from healthy control dogs in the present study appeared to be similar to those described in similar populations of some other studies, despite the use

<table>
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<tr>
<th>Sample type</th>
<th>No. evaluated</th>
<th>No. in agreement</th>
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<th>Negative flow cytometric and positive cytologic examination results</th>
<th>Negative flow cytometric and suspicious cytologic examination results</th>
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<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>5</td>
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<td>3</td>
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<td>3</td>
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</tr>
<tr>
<td>All</td>
<td>161</td>
<td>133</td>
<td>11</td>
<td>8</td>
<td>9</td>
<td>28</td>
</tr>
</tbody>
</table>

Fifteen of 176 samples were excluded from analysis (11 had insufficient cellularity for flow cytometric evaluation and 4 were not of acceptable quality for cytologic evaluation). Mean values of the log(T:B) = 2 SD in healthy control dogs were used to define upper and lower threshold values for each sample type; for flow cytometric evaluation, samples were considered positive for lymphoma involvement if the log(T:B) was less than the lower threshold value in dogs with B-cell lymphoma or greater than the upper threshold value in dogs with T-cell lymphoma. In cytologic evaluations, peripheral blood, bone marrow, or spleen samples considered positive contained > 1%, > 2%, or ≥ 5% lymphoma cells, respectively; liver samples considered positive contained ≥ 5 lymphoma cells/hpf at 400× magnification. Suspected extranodal lymphoma involvement was determined if percentages of suspected lymphoma cells were less than the described values or if a subjectively large number of blast cells of different lineages were detected.
of different sample collection methods.\textsuperscript{24-26} For example, investigators in 1 study\textsuperscript{26} reported a mean ratio of CD3-positive–to–CD21-positive cells of 2.1 in spleen samples from 3 healthy Beagles, whereas the mean T:B identified in spleen samples of 5 healthy Beagles in the present study was 2.3.

None of the previous flow cytometric studies\textsuperscript{24-26,29} performed to evaluate lymphocytes in samples of canine liver, spleen, or both used cells obtained via in vivo fine-needle aspiration, although flow cytometric examination of fine-needle aspirates is an established method for evaluation of lymph node samples from dogs with and without lymphoma.\textsuperscript{38,41} In addition to blood and bone marrow samples in the present study, we evaluated lymph node, liver, and spleen samples obtained via fine-needle aspiration. The majorities of all samples collected (233/245 [95%]) and of liver and spleen fine-needle aspirates (88/98 [90%]) were suitable for flow cytometry.

In human medicine, collection of fine-needle aspirate samples from abdominal organs and subsequent flow cytometric analysis is an established technique in patients with NHL.\textsuperscript{30-33} Scarcity of cells has previously been reported for samples collected by use of this method.\textsuperscript{31,32,34} In the present study, most of the samples that were excluded because of low cellularity were obtained and analyzed during the first year of the 2-year study period. This difference may be attributable to learned changes in technique among the personnel involved in the sample collection procedure. A similar effect was observed in a flow cytometry–based study\textsuperscript{32} in which samples from human patients were collected via fine-needle aspiration. Furthermore, in the present study, the median cellularity of liver samples in dogs with lymphoma (2,766 mononuclear leukocytes) was considerably higher than that in healthy control dogs (1,229 mononuclear leukocytes), despite the use of identical sample collection techniques. A possible explanation for this observation is that lymphoma-infiltrated tissue contains more cells that are easily aspirated (ie, the infiltrating lymphoma cells) than does normal hepatic tissue, which mainly comprises epithelial cells.

Flow cytometry has gained an important role in the diagnostic testing of human patients with hematolymphoid neoplasias, including NHL.\textsuperscript{33,38} It is an established method for disease staging in NHL, especially as an ancillary test; when used in combination with morphological evaluation, it can enhance diagnostic precision and provide additional information regarding the extent of disease spread.\textsuperscript{31,37,38}

One advantage of flow cytometric evaluation of samples from humans with B-cell lymphoma is that determination of B-cell clonality can be used to detect neoplastic cells. Given that the detection of immunoglobulin light-chain class restriction (\(\kappa\) or \(\lambda\)) is an indicator of clonality, even small numbers of neoplastic cells can be recognized in samples.\textsuperscript{31} Use of flow cytometry to evaluate T-cell lymphomas in humans is more complicated, but identification of neoplastic T cells can be aided by the detection of aberrant antigen expression (a marker of pseudoclonality),\textsuperscript{39} which requires the use of wide arrays of antibodies.

In dogs with lymphoma, flow cytometric detection of neoplastic cells is more difficult. No lymphoma-specific antigen is known, and flow cytometric detection of clonality via light chain restriction is not possible in dogs with B-cell lymphomas because of a wider \(\kappa\)-to-\(\lambda\) ratio,\textsuperscript{40} compared with that of humans. A possible method for detection of pseudoclonality in dogs was identified in studies\textsuperscript{20,41} that revealed aberrant antigen expression in dogs with B-cell and T-cell lymphomas.

In the present study, the log(T:B) was used to discriminate between lymphoma-infiltrated and healthy tissue samples. The T:B objectively reflects proportions of these distinct lymphocyte phenotypes. Because values of log(T:B) were normally distributed for all sample types from healthy control dogs as well as those with T-cell lymphoma and B-cell lymphoma, they were used to define thresholds for flow cytometric determinations. Small amounts of infiltrating lymphoma cells may not be detected with this method. Conversely, false-positive classifications are possible (eg, when pathological conditions other than lymphoma cell infiltration alter the lymphocyte subset distribution in the sample collection site). On flow cytometric dot plots of dogs with B-cell lymphoma, lymphocyte populations were detected in blood, bone marrow, spleen, and liver samples that had light scatter and antibody binding characteristics similar to those of the lymphoma cell population in the lymph node sample of the same patient. These were presumed to be infiltrating lymphoma cells, but because a specific lymphoma cell marker was not available, this can only be an assumption. It cannot be ruled out that these populations represent lymphocyte subpopulations occurring in the respective site physiologically or in response to other, nonneoplastic pathological conditions. For example, the 2 populations of B cells (distinguished on the basis of high or low relative CD21 expression density) observed in spleen samples of healthy control dogs and of many dogs with T-cell lymphoma represent lymphocyte subsets with distinct immunophenotypic and light scatter characteristics. In the available literature regarding use of flow cytometry to evaluate spleen samples from dogs, no evidence of distinct population formation on the basis of CD21 staining has been described. Because FSC and SSC properties of B-cells with high CD21 expression density were subjectively higher than those of B-cells with low CD21 expression density in the present study, it could be assumed that the former cell population represents larger lymphocytes naturally occurring in the spleen. Further flow cytometric analysis by use of a wider array of antibodies or immunohistochemical studies may further characterize different populations of CD21-positive cells in the spleen. However, lymphocyte subsets like these can be difficult to distinguish from lymphoma cell populations in flow cytometric examinations, particularly in tissues other than lymph nodes.

In the present study, presumed lymphoma cell populations of 24 of 35 (69%) dogs with B-cell lymphoma had higher relative CD21 expression density than did apparently normal B cells in the same dogs in subjective evaluation of flow cytometry data. Diminished CD21 expression, reported in 2 of 31 dogs with
B-cell lymphoma in another study, was not detected in the present study. The semiquantitative approach used in that study detected higher CD21 expression in the neoplastic cells of only 1 dog with B-cell lymphoma; in 16 dogs with T-cell lymphoma, the neoplastic cells of 1 had diminished CD3 expression, and in 2 dogs, they lacked CD3 expression. Among dogs with T-cell lymphoma in the present study, no aberrant CD3 expression was observed; however, expression of CD3 was a study inclusion requirement for dogs with T-cell lymphoma.

Significant differences were detected in mean log(T:B) values between dogs with B-cell and T-cell lymphoma and between dogs with B-cell lymphoma and healthy control dogs at all sample sites in the present study. When comparing mean log(T:B) values between dogs with T-cell lymphoma and healthy controls, significant differences were found in lymph node, bone marrow, and spleen samples but not in blood or liver samples. The lack of significant differences in some sample types between dogs with T-cell lymphoma and healthy control dogs was most likely caused in part by a predominance of CD3-positive over CD21-positive lymphocytes that has been reported for many tissues in nonneoplastic conditions; evidence of this was detected in healthy control dogs of the present study as well as in previous studies and this might have led to an underestimation of samples considered positive for extranodal lymphoma involvement in dogs with T-cell lymphoma in the present study. However, results of cytologic evaluation supported the finding that the proportion of sites with extranodal lymphoma involvement in T-cell lymphoma–affected dogs in this study was smaller than that in B-cell lymphoma–affected dogs. Another interesting observation of the present study was that a wide range of T:B values was detected in samples of lymphoma-affected dogs. Whether the T:B (as an indicator of the degree of lymphoma cell infiltration) could serve as prognostic marker in dogs with lymphoma remains to be investigated in future studies.

Comparison of results of flow cytometric evaluation to those of cytologic examination showed agreement regarding extranodal lymphoma involvement (positive or negative) in 133 of 161 (83%) sample collections sites. The 28 (17%) sites in which disagreement was detected were not equally distributed among sample sites. Although numbers were small and values were not statistically compared, the percentage of disagreements appeared to be greatest in bone marrow samples. This may be attributable to a high variance in the log(T:B), which was detected in bone marrow samples from healthy control dogs (ie, these had the highest SD of sample types). It can be assumed that this increased the number of false-negative classifications and led to more disagreement in results between the 2 methods. This assumption was further substantiated by the fact that 13 of the 17 disagreements in bone marrow samples were considered negative for extranodal lymphoma involvement via flow cytometry and positive or suspected for lymphoma involvement via cytologic examination.

Limitations of the present study should be considered in evaluating these findings. The number of dogs, particularly healthy controls, was small. A larger number of healthy controls might have led to more precise thresholds, resulting in a more reliable flow cytometric classification of samples. Furthermore, only a small number of antibodies were used. Use of a wider array of antibodies might result in more specific information about the neoplastic cells and the lymphocyte subset distribution in dogs with lymphoma. Further studies are indicated.

Another issue that should be addressed is the aspect of blood contamination, which might lead to false classifications in both cytologic and flow cytometric examination. When neoplastic cells are present in the blood, it cannot be ruled out that neoplastic cells found in fine-needle aspirate samples of other tissue types are attributable to contaminating blood. Because increased exfoliation of (lymphoma) cells in lymphoma-infiltrated tissue can be assumed, blood contamination could also correlate negatively with lymphoma involvement (ie, when fewer cells from the sampled tissue exfoliate, more blood contaminates the sample). This would further contribute to the problem of false classification caused by detection of lymphoma cells from contaminating blood. Regarding the lymphoma staging system, this would not alter staging of lymphoma in patients because involvement of blood or bone marrow results in assignment to the highest stage (stage V). The questions of whether the addition of flow cytometry to existing diagnostic techniques will lead to considerable stage migration and whether the identification of previously undetected extranodal lymphoma involvement in certain sites is of clinical relevance as a prognostic indicator merit further research.

In the present study, we determined that blood and bone marrow samples and fine-needle aspirate samples collected from the liver and spleen of dogs with and without lymphoma were suitable for use in flow cytometry. Taking the described limitations into account, a flow cytometric diagnosis of neoplastic cell infiltration in extranodal sites of canine lymphoma patients by use of thresholds for lymphocyte subset ratios seems possible, although definitive identification of lymphoma cells in dogs via flow cytometry is still challenging. These encouraging results support the need for more extensive studies, including investigation of a wider array of antibodies.

References


