Prevalence and prognostic impact of hypocobalaminemia in dogs with lymphoma

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Objective—To determine the prevalence of hypocobalaminemia in dogs with multicentric lymphoma and to investigate any relationship between serum cobalamin concentration and disease outcome.

Design—Cohort study.

Animals—58 dogs with multicentric lymphoma.

Procedures—Serum cobalamin concentrations were measured in 58 dogs with multicentric lymphoma. Clinical signs, stage, and immunophenotype for dogs with hypocobalaminemia were compared with those for dogs with serum cobalamin concentrations above the lower end of the reference range. Survival times for dogs undergoing a cyclic multidrug chemotherapy protocol (n = 53) were similarly compared. Serum cobalamin concentrations for treated dogs that died or were euthanized before day 60 were compared with those of dogs still alive at day 60.

Results—Serum cobalamin concentrations ranged from <150 to 1,813 ng/L, with a median concentration of 401 ng/L. Nine of the 58 (16%) dogs had hypocobalaminemia (serum cobalamin concentration <252 ng/L). Three of 9 dogs with hypocobalaminemia survived to at least day 60, compared with 40 of 44 (91%) dogs without hypocobalaminemia (serum cobalamin concentration ≥252 ng/L). Ten (10/53 [19%]) dogs undergoing a cyclic multidrug chemotherapy protocol died before day 60, and the median serum cobalamin concentration for these dogs (232 ng/L) was significantly lower than for those still alive at the end point of the study (556 ng/L).

Conclusions and Clinical Relevance—Hypocobalaminemia was relatively uncommon in this population of dogs with multicentric lymphoma, but was associated with a poor outcome. Serum cobalamin concentrations may provide prognostic information in dogs with multicentric lymphoma. (J Am Vet Med Assoc 2009;235:1437–1441)

Cobalamin (vitamin B₁₂) is an essential cofactor for 3 mammalian enzyme systems, and adequate amounts are required for nucleic acid synthesis and hematopoiesis. Dogs are unable to synthesize cobalamin and are entirely dependent upon adequate dietary sources, principally organ and muscle tissues of herbivores. The absorption of cobalamin is complex, as it is first bound to R-protein, then to gastric or pancreatic intrinsic factor, and finally transferred to specific receptors located on the enterocyte of the ileum. Hypocobalaminemia can develop for several reasons, including exocrine pancreatic insufficiency, changes in small intestinal bacterial populations, and diffuse disease of the distal portion of the small intestine. The Association of American Feed Officials’ compliant pet foods contain adequate amounts of cobalamin, so dietary deficiency is unlikely. Serum cobalamin concentrations are routinely measured in dogs with known or suspected gastrointestinal tract disease, and hypocobalaminemia has been associated with a poor outcome in dogs with chronic enteropathies. In addition, it has recently been suggested that hypocobalaminemia may directly compromise gastrointestinal tract function in cats with intestinal disease and influence the response to other treatments.

Low serum cobalamin concentrations were documented for 78% (23/32) of cats with low-grade lymphocytic lymphoma, but have not been previously reported for dogs with multicentric lymphoma. In the study on cats, every patient with hypocobalaminemia had histologically confirmed lymphoma in the small intestine. We hypothesized that a subset of dogs with multicentric lymphoma may have hypocobalaminemia as a result of intestinal infiltration and that hypocobalaminemia may therefore provide useful prognostic information. The goals of the study presented here were to determine the prevalence of hypocobalaminemia in dogs with multicentric lymphoma; compare this with clinical signs, disease stage, and immunophenotype; and investigate any relationship between serum cobalamin concentration and patient outcome following chemotherapy.
Materials and Methods

Animals—Dogs included in this study were evaluated for multicentric lymphoma at the Veterinary Medical Teaching Hospital at the College of Veterinary Medicine and Biomedical Sciences at Texas A&M University; College Station, Tex, or the Veterinary Specialty Center of Texas in Round Rock, Tex, between September 2005 and July 2007. The diagnosis of lymphoma was made by cytologic or histologic examination of affected tissues. None of these patients had received any chemotherapeutic medications (except for corticosteroids), and food was withheld from all dogs for at least 12 hours prior to initial examination.

Sample collection—All serum samples were surplus from an earlier project that involved examining the effects of chemotherapy on markers of systemic inflammation. These samples were collected during the diagnostic and staging processes and prior to the administration of any injectable chemotherapeutic agents. Serum samples were maintained at ~80°C for up to 18 months before measurement of cobalamin concentrations. All aspects of the original study were approved by the Clinical Research Review Committee at Texas A&M University College of Veterinary Medicine and Biomedical Sciences, and informed client consent was obtained.

Serum cobalamin measurement—An automated chemiluminescence assay was performed at the Gastrointestinal Laboratory at Texas A&M University. This assay was validated in-house with the following validation variables: mean ± SD coefficients of variation for intra-assay and interassay variability were 3.0 ± 0.2% and 3.7% ± 2.4%, respectively. The observed-to-expected ratio for serum dilutions for 4 serum samples ranged from 92.9% to 103.3%. Samples were evaluated in several batches, and the appropriate controls were performed each time. The reference range had been previously determined by calculation of the central 95th percentile of serum cobalamin concentration in 120 healthy pet dogs and was determined to be 252 to 908 ng/L. Dogs were classified as having hypocobalaminemia if the serum cobalamin concentration was < 252 ng/L.

Hematologic variables—Red blood cell and WBC counts were determined by use of an automated counter, and the WBC differential was confirmed with a manual 100-cell count. Only hematologic findings from blood samples collected within 24 hours of blood samples used for determination of serum cobalamin concentration were included in the data analysis.

Immunophenotyping and staging—Immunophenotyping was performed on fine-needle aspirates of affected tissue by use of antibodies against CD79a and CD3. All patients were classified as staged by an American College of Veterinary Internal Medicine board-certified oncologist or an oncology resident prior to the administration of any injectable chemotherapeutic agents. Numerical stage was determined by anatomic location of disease (Appendix). The substage reflected the absence (substage a) or presence (substage b) of clinical illness (eg, anorexia, vomiting, diarrhea, lethargy, weakness, or dyspnea). Staging methods included physical examination, CBC, thoracic and abdominal radiography, abdominal ultrasonography, and cytologic evaluation of bone marrow specimens. Decisions regarding staging were made by the supervising oncologist, and not all procedures were performed in all patients.

Clinical status and prior corticosteroid administration—Medical records were reviewed for information about anorexia, vomiting, or diarrhea prior to the initial visit. Information on the dose and duration of corticosteroids administered in the 4 weeks prior to the initial visit was solicited from the pet owner and verified by review of medical records of the referring veterinarian.

Survival times—Survival data were evaluated for all dogs undergoing a cyclic multidrug chemotherapy protocol (ie, cyclophosphamide, i-asparaginase, vincristine, doxorubicin, and prednisone). The date of death, euthanasia, or completion of the study (set at day 60 from initial admission) was determined by review of the medical record or telephone contact with the referring veterinarian. Survival data from patients receiving only antineoplastic drugs PO were not included in this portion of the study.

Statistical analysis—Statistical software was used for data analysis. Serum cobalamin concentrations below the working range of the assay of 150 ng/L were assumed to be 149 ng/L. Data sets were evaluated for normality by use of a Kolmogorov-Smirnov test. Categorical data were compared between groups by use of the χ² test or the Fisher exact test. Survival times were evaluated by use of Kaplan-Meier methods, and patient groups were compared by use of a log rank test. Serum cobalamin concentrations of dogs that died before day 60 or survived to the endpoint of the study were compared by use of a Mann-Whitney U test. Values of P < 0.05 were considered significant.

Results

Animals—Samples for serum cobalamin measurement were available for 58 dogs. The median age was 8 years old, with a range of 2 to 16 years old. Twenty-nine dogs were spayed females, 21 were castrated males, and 8 were sexually intact males. Twenty-one purebred dogs were represented, and 16 dogs were of mixed breeding. Boxers (n = 6) and Golden Retrievers (5) were the most prevalent purebreds.

 Serum cobalamin concentrations—The range of serum cobalamin concentrations was < 150 ng/L (lower detection limit of assay) to 1,813 ng/L, with a median concentration of 401 ng/L. Nine of the 58 (16%) dogs had hypocobalaminemia, with serum cobalamin concentrations below the established reference range of 252 to 908 ng/L.

Hematologic variables—A contemporaneous CBC was available for 51 dogs (9/9 dogs with hypocobalaminemia; 42/49 dogs without hypocobalaminemia [serum cobalamin concentration ≥ 252 ng/L]). The RBC counts ranged from 1.95 × 10⁶ RBCs/µL to 8.47 × 10⁶ RBCs/µL, with a mean of 5.84...
× 10⁶ RBCs/μL (reference range, 5.5 × 10⁶ RBCs/μL to 8.5 × 10⁶ RBCs/μL). Neutrophil counts ranged from 1,500 to 27,740 cells/μL, with a mean of 10,993 cells/μL (reference range, 3,000 to 11,500 cells/μL). There were no significant differences between hematologic findings for dogs with and without hypocobalaminemia. Mean RBC counts were similar for dogs with and without hypocobalaminemia (5.82 × 10⁶ RBCs/μL and 5.85 × 10⁶ RBCs/μL, respectively); 2 of the 9 dogs with hypocobalaminemia and 10 of the 42 (24%) dogs without hypocobalaminemia were anemic (RBC count < 5.5 × 10⁶ RBCs/μL). Mean neutrophil count for dogs with hypocobalaminemia was slightly higher than for dogs without hypocobalaminemia (11,826 cells/μL vs 10,751 cells/μL, respectively), but this difference was not significant. None of the dogs were neutropenic when first evaluated.

Immunophenotyping—Results were available for 32 of the 58 dogs. Overall, B-cell lymphoma (12/32 [38%]) was more prevalent than T-cell lymphoma (10/32 [31%]). T-cell lymphomas were more prevalent in dogs with hypocobalaminemia than in dogs without hypocobalaminemia (3/6 dogs vs 7/26 [27%] dogs, respectively), but this finding was not significant.

Staging—Staging methods varied among dogs and between institutions. Abdominal ultrasonography was performed on 7 of 9 dogs with hypocobalaminemia, but only 13 of 49 (27%) dogs without hypocobalaminemia; gastrointestinal tract involvement was not observed in any dogs that underwent abdominal ultrasonography (Table 1). Overall, most dogs (24/58 [41%]) were classified as stage 3 and most were clinically well (substage a; 35/58 [60%]). Although a higher percentage of dogs with hypocobalaminemia were classified as stage 5, this was not a significant finding. However, dogs with hypocobalaminemia were significantly more likely to be classified as substage b (7/9 dogs) than dogs without hypocobalaminemia (16/49 [33%] dogs).

Clinical status—Health information regarding the gastrointestinal system (eg, appetite, stool consistency, and vomiting) was available for all 58 dogs. A total of 15 (26%) dogs had signs suggesting gastrointestinal tract disease. Anorexia was the most common complaint and vomiting and diarrhea were observed in 3 dogs. Signs of gastrointestinal tract disease were commonly observed in dogs with hypocobalaminemia, with 4 of 9 reportedly anorexic (with or without vomiting and diarrhea); however, this finding was not significantly different from that of dogs without hypocobalaminemia (11/49 [22%]).

Prior corticosteroid administration—Fourteen of the 58 (24%) dogs received corticosteroids in the 4-week period prior to initial evaluation. Duration of treatment ranged from 1 to 30 days (median, 7 days). The incidence of prior corticosteroid administration was similar between dogs with and without hypocobalaminemia (2/9 dogs vs 12/49 [24%] dogs, respectively).

Survival times—Five dogs were treated PO only with antineoplastic drugs and were therefore excluded from this portion of the study. The remaining 53 dogs underwent a cyclic multidrug chemotherapy protocol, and survival data (up to day 60) were evaluated for these dogs. All 9 dogs with hypocobalaminemia started a cyclic multidrug chemotherapy protocol, but only 3 were still alive at the endpoint of the study (day 60); 40

### Table 1—Staging classification for 58 dogs with multicentric lymphoma and with (9 dogs) and without (49 dogs) hypocobalaminemia*

<table>
<thead>
<tr>
<th>Stage</th>
<th>No. of all dogs</th>
<th>With hypocobalaminemia</th>
<th>Without hypocobalaminemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>1</td>
<td>1/1 (100%)</td>
<td>0/1 (0%)</td>
</tr>
<tr>
<td>2b</td>
<td>2</td>
<td>1/2 (50%)</td>
<td>0/2 (0%)</td>
</tr>
<tr>
<td>3a</td>
<td>19</td>
<td>2/19 (11%)</td>
<td>17/49 (35%)</td>
</tr>
<tr>
<td>3b</td>
<td>5</td>
<td>0</td>
<td>5/49 (10%)</td>
</tr>
<tr>
<td>3c</td>
<td>8</td>
<td>0</td>
<td>8/49 (16%)</td>
</tr>
<tr>
<td>4a</td>
<td>8</td>
<td>2/8 (25%)</td>
<td>6/41 (19%)</td>
</tr>
<tr>
<td>4b</td>
<td>8</td>
<td>0</td>
<td>7/49 (14%)</td>
</tr>
<tr>
<td>5a</td>
<td>7</td>
<td>4/7 (57%)</td>
<td>3/42 (7%)</td>
</tr>
<tr>
<td>5b</td>
<td>8</td>
<td>4/8 (50%)</td>
<td>4/40 (10%)</td>
</tr>
</tbody>
</table>

*Dog classified as having hypocobalaminemia if serum cobalamin concentration was < 252 ng/L.
of 44 (91%) dogs without hypocobalaminemia survived to day 60 (P < 0.001 for survival rate between groups; Figure 1).

Forty-three of the 53 (81%) dogs undergoing a cyclic multidrug chemotherapy protocol were alive at day 60. Median serum cobalamin concentration for these 43 dogs (356 ng/L) was significantly (P < 0.001) higher than that for the 10 dogs (232 ng/L) that died or were euthanized before day 60 (Figure 2).

Discussion

Hypocobalaminemia was relatively uncommon in this population of dogs with multicentric lymphoma, but was significantly associated with a poor outcome in dogs undergoing a cyclic multidrug chemotherapy protocol. In addition, the median serum cobalamin concentration was significantly higher in the dogs still alive at day 60.

Cobalamin is a water-soluble vitamin and is an essential cofactor for 3 mammalian enzyme systems, including methionine synthetase. This enzyme is required for nucleic acid synthesis, cell growth, and hematopoiesis. Consequently, rapidly dividing cells are particularly vulnerable to compromise if adequate amounts of this vitamin are not available. Clinical signs reported for dogs with inherited selective cobalamin deficiency include failure to thrive, poor appetite, and hematologic changes, including neutropenia and anemia. The clinical impact of acquired hypocobalaminemia in dogs has not been well described, but hematologic abnormalities have not been reported and were not evident in this study population. This may be attributed to a shorter duration of disease or other unidentified influences. Prolonged cobalamin deficiency in people causes widespread pathological changes, with demyelination and gastrointestinal compromise. The latter is characterized by increased intestinal permeability and diminished absorptive capacity; these abnormalities are reversed when cobalamin concentrations are restored.

Acquired cobalamin deficiency is an established consequence of chronic gastrointestinal tract disease in dogs and cats, but it has recently been suggested that hypocobalaminemia may directly impact intestinal health and function in affected cats.

We did not attempt to determine the cause of hypocobalaminemia in this study population. The most common etiologies of hypocobalaminemia in dogs are exocrine pancreatic insufficiency and severe infiltrative or inflammatory disease in the distal portion of the small intestine. Unfortunately, we were unable to evaluate exocrine pancreatic function or intestinal histopathologic changes in any of the dogs, so firm conclusions about the cause of hypocobalaminemia cannot be made. It is also possible that hypocobalaminemia in dogs with lymphoma reflects sequestration or rapid use of cobalamin by neoplastic cells and does not indicate gastrointestinal tract dysfunction. This has not been reported for dogs or any other species, and it therefore seems more likely that hypocobalaminemia was caused by infiltration of the ileum by neoplastic lymphocytes, resulting in compromised gastrointestinal uptake.

At present, abdominal ultrasonography appears to be the most appropriate noninvasive method for identifying gastrointestinal tract involvement in dogs with multicentric lymphoma. Although changes in wall thickness or architecture have been reported for dogs with intestinal infiltration, ultrasonography is likely to be an insensitive method for detecting disease. Patient staging is substantially impacted by this limitation, as intestinal involvement conveys a stage 5 designation. If we conclude that hypocobalaminemia likely indicates gastrointestinal tract involvement, measurement of serum cobalamin concentration may be a safe and inexpensive adjunct to conventional staging protocols. Dogs with confirmed lymphoma in the small intestine generally merit a poor prognosis, with a median reported survival time of just 13 days.

The cause of death or reason for euthanasia before day 60 was not investigated in this group of dogs. These patients may have been refractory to treatment, had substantial morbidity from chemotherapy (eg, gastroenteritis and sepsis), or simply been judged by their owners to have a poor quality of life. Consequently, we cannot draw any definitive conclusions regarding the association between serum cobalamin concentrations and patient survival rate, and it is probable that several factors played a role. The hypocobalaminemia may have directly affected patient appetite and attitude, causing owners to question the benefits of further treatment. It is also possible that inadequate serum cobalamin concentrations somehow mitigated the antineoplastic effects of chemotherapy or compromised the bone marrow response during treatment. Alternatively, preexisting gastrointestinal tract dysfunction, combined with compromised enterocyte metabolism secondary to chemotherapy, may have resulted in increased morbidity in dogs with hypocobalaminemia.

There are several limitations to this retrospective study. Information regarding parenteral cobalamin supplementation prior to referral was not available, and we did not determine whether any of the dogs received cobalamin supplementation after their initial examination. Oral administration of cobalamin is not expected to improve serum cobalamin concentrations in dogs with absorptive compromise, but parenteral administration of cobalamin would certainly have an impact. In addition, we were unable to measure serum cobalamin concentrations at other time points to see whether chemotherapy impacted this variable. It is also important to point out that serum cobalamin concentrations may not accurately reflect events at the cellular level and that measurement of cobalamin-dependent metabolites, such as methylmalonic acid, may be more sensitive in reflecting cobalamin status. In people with pernicious anemia, these metabolites are often high before serum cobalamin concentrations decrease below the reference range. Conversely, up to 40% of elderly people with low serum cobalamin concentrations have a normal metabolic function and are therefore not truly cobalamin deficient. Some of these individuals may simply have low amounts of cobalamin carrier molecules; this possibility has not been evaluated in dogs. All dogs with hypocobalaminemia had died before their serum cobalamin concentrations were measured, so we were unable to determine the impact of appropriate supplementation. Future studies are needed to investigate the cause of hypocobalaminemia in dogs with multicentric lymphoma.
determine the effect of parenteral supplementation in hypocobalaminemic individuals.

In conclusion, hypocobalaminemia was strongly associated with a poor outcome in dogs with multicentric lymphoma enrolled in this study. Although many factors have been shown to impact prognosis, including anatomic involvement (denoted by the numerical stage), the presence or absence of clinical disease (denoted by subtype), phenotype (B or T lymphocyte), and histologic grade, patient survival rate remains unpredictable. This study indicates that serum cobalamin concentrations provide additional prognostic information, and determination of serum cobalamin concentration merits consideration as part of the routine evaluation of dogs with multicentric lymphoma.

References

Appendix
Staging criteria used for 58 dogs with lymphoma.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Anatomic location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Single node</td>
</tr>
<tr>
<td>2</td>
<td>Two or more node regions; same side of the diaphragm</td>
</tr>
<tr>
<td>3</td>
<td>Two or more node regions; different sides of the diaphragm</td>
</tr>
<tr>
<td>4</td>
<td>Any nodes plus liver or spleen</td>
</tr>
<tr>
<td>5</td>
<td>Extranodal tissue involvement (eg, bone marrow, eye, lung, kidney, and intestine)</td>
</tr>
</tbody>
</table>