Invited Review

Molecular methods to distinguish reactive and neoplastic lymphocyte expansions and their importance in transitional neoplastic states

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Abstract: Although lymphoma and leukemia usually can be diagnosed by routine cytology and histology, some cases present a diagnostic challenge for pathologists and clinicians. Often the dilemma lies in determining whether a population of lymphocytes is reactive or neoplastic. We review currently available methods for analyzing lymphocyte populations by immunophenotyping and by identifying clonally rearranged immunoglobulin and T-cell receptor genes and discuss how these tests can be used to clarify such diagnostic dilemmas. We also describe the detection of chromosomal abnormalities and methods on the horizon, such as gene expression profiling, to identify diagnostically useful oncogenes. Finally, we review the emerging concept of transitional neoplastic states, in which reactive lymphocytes transform to neoplastic lymphocytes in the presence of continued antigenic stimulation, such as that caused by infection with *Helicobacter pylori*. The existence of transitional neoplastic states underscores the need for an array of molecular diagnostic tools that would improve our ability to characterize lymphocyte populations in human and animal patients and enhance early detection of neoplastic lymphocytes such that eradication of the infectious or inflammatory stimulus could lead to cure. (*Vet Clin Pathol*. 2004;33:196–207)

Key Words: Clonality assessment, diagnosis, gene expression profiling, immunophenotyping, lymphoma, veterinary

Introduction

Lymphoma and lymphocytic leukemia are derived from heterogeneous cells. As such, they give rise to a diverse set of clinical signs and syndromes. These diseases also present some of the most commonly encountered diagnostic dilemmas faced by clinicians and pathologists because reactive lymphocytes can be difficult to distinguish from neoplastic lymphocytes. A vigorous, polyclonal lymphocyte proliferation can be induced by infection, autoimmune disorders, genetic diseases involving failure of lymphocyte homeostasis, and certain types of malignancies. Lymphocyte expansions can present a confusing clinical and morphologic picture, the diagnosis of which can result in opposing treatment strategies, ie, chemotherapy with resultant immunosuppression in the case of neoplasia or antimicrobial therapy in the case of infection. The distinction between reactive and neoplastic lymphocytes, therefore, has substantial clinical importance.

As diagnosticians, we often are presented with a peripheral blood film or with aspirated material from an enlarged lymph node, lymphoid organ, or bone marrow and asked to distinguish between reactive, inflammatory, and neoplastic states. Many cases of canine lymphoma can be diagnosed by cytology alone. All pathologists, however, have been confronted with cases in which atypical features or subtle expansions of

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lymphocyte populations do not quite satisfy the typical criteria for a diagnosis of lymphoma. Incipient or small-cell lymphoma in dogs and Hodgkin's-like lymphoma in cats[1] are examples of this situation.

Relying on cellular atypia to define lymphoid malignancies in animals can delay diagnosis and may result in decreased detection of indolent forms of lymphoma, which have been well-characterized in people. Differentiating benign from neoplastic expansions of lymphocytes can be a problem in particular clinical situations. Specific examples of cytologic or histologic dilemmas in veterinary medicine include circulating large granular lymphocytes (LGLs), which can be seen both in canine *Ehrlichia canis* infection and in leukemia[2–4]; lymphocyte-rich thoracic effusion in the presence of a mediastinal mass, which can suggest chylous effusion or exfoliation from small-cell lymphoma or thymoma; lymphoblast predominance in splenic aspirates, which can suggest either a sample from the germinal center of a lymphoid follicle or lymphoma; inflammatory bowel disease and intestinal lymphomas, which share many cytologic and histologic features; and an increased number of plasma cells in a bone marrow sample, which can be seen with sustained antigenic stimulation, eg, due to *E canis* infection, but which also is a hallmark of multiple myeloma. Such cases point to the importance of using and expanding the techniques that can distinguish between benign and malignant lymphocyte expansions in animals.

A number of methods can be used to aid in distinguishing between reactive and neoplastic lymphocyte populations: 1) demonstrating a uniform or aberrant immunophenotype, 2) establishing cellular clonality, 3) identifying chromosomal abnormalities, and 4) identifying the presence of an oncogene associated with malignancy. The first 2 methods are readily available for most veterinary patients. The latter 2 methods are less well developed and not routinely available; however, the full sequence of the canine genome[5] and work by Breen and colleagues[6] to develop molecular methods of examining chromosomal aberrations will facilitate the development of future diagnostic assays. The purpose of this review is to discuss each of these approaches for distinguishing reactive from neoplastic lymphocyte populations. In addition, we review transitional states in animals and humans that illustrate the importance of early and sensitive differentiation of lymphocyte expansions.

Molecular Indicators of Malignancy

Immunophenotyping

One of the most readily available methods for distinguishing reactive from neoplastic lymphocyte populations is immunophenotyping, which can establish the degree of lymphocyte diversity within a population and ascertain whether the cells express a normal constellation of surface markers. A phenotypically homogeneous population of lymphocytes suggests a neoplastic rather than a reactive process. For example, canine chronic lymphocytic leukemia (CLL) most commonly involves an expansion of CD8+ T cells and, less frequently, B cells.[7–9] CD8+ T cells usually comprise 25–35% of canine peripheral blood lymphocytes, whereas B cells usually comprise 5–20%.[10,11] Therefore, leukemia would be the primary differential diagnosis in a dog with lymphocytosis when a majority of peripheral blood lymphocytes are CD8+ T cells or B cells, although objective criteria for making this diagnosis have not yet been established in veterinary medicine. The caveat to such an interpretation is that infectious or inflammatory diseases can also, on rare occasions, result in expansion of a phenotypically homogeneous population of lymphocytes. *E canis* infection, which is associated with the expansion of CD8+ T cells that have LGL morphology, is the only well-documented example of this in dogs[2–4,12] and, to our knowledge, there are no examples in cats. Studies of the predictive value of expanded lymphocyte populations and absolute lymphocyte counts for diagnosing leukemia would be important and clinically useful, but at present, no such information is available in the veterinary literature.

Uniform lymphocyte expansion at other sites can be interpreted similarly to those in blood. Because phenotypic analysis of normal lymphocytes in the thoracic duct of sheep, mice, and humans has shown that 75–90% of the lymphocytes are T cells,[13,14] pleural fluid that contains a majority of B cells supports a diagnosis of B-cell lymphoma rather than chylothorax. To our knowledge, the immunophenotypic characterization of pleural fluid from dogs and cats has not been described. Thus, although certain diseases can result in expansion of a single lymphocyte phenotype, a predominance of one lymphocyte subset in the absence of infectious or inflammatory disease supports a diagnosis of malignancy.

Aberrant antigen expression can provide a definitive diagnosis of leukemia or lymphoma[15] because reactive lymphocytes generally retain expression of the normal constellation of antigens. For example, human T cells do not lose expression of CD4, CD8, or other T-cell markers when they expand, except in certain types of inherited human lymphoproliferative disorders, such as autoimmune lymphoproliferative syndrome.[16] Therefore, the finding that a significant population of T cells has lost expression of one or more T-cell markers is strong support for a diagnosis of lymphoma or leukemia.

Human T-cell leukemia is characterized by its tendency to lose expression of normal T-cell antigens

or to express aberrant combinations of antigens.[17] In one study of 87 humans with malignant T-cell disorders, Gorczyca et al found that complete loss of any T-cell antigen (CD2, CD5, CD7) or the pan-leukocyte antigen CD45 was diagnostic for malignancy.[18] In that case series, a small percentage of inflammatory conditions, such as Epstein-Barr virus infection, was characterized by a subset of T cells with reduced expression of some antigens but none with complete loss. In studies of occult intestinal lymphoma in people, the presence of T cells that expressed CD3 but not CD4 or CD8 was a useful diagnostic marker for distinguishing lymphoma from inflammatory bowel disease.[19]

Although the immunophenotypic markers of canine lymphoma and leukemia have been described in a number of studies,[4,7,9,20–26] aberrant antigen expression has not been reported. To detect aberrant antigen expression, it is useful to examine a large panel of antigens using multicolor fluorescence protocols. In our experience, almost half (11 of 26) of the T-cell leukemias phenotyped during a 1-year period exhibited aberrant antigen expression (no expression of CD4 or CD8, loss of CD45, occasional loss of CD5). In one case (Figure 1), a dog with 30,000 lymphocytes/μL in peripheral blood but normal lymphocyte morphology had a substantial number of CD3+ T cells that failed to express CD45, CD4, or CD8. The lymphocytes did express the pan–T-cell marker CD5, and using polymerase chain reaction (PCR) for antigen-receptor rearrangements (PARR), a clonal T-cell population was detected. Even without immunophenotypic data, there may have been little doubt as to the diagnosis in this case, yet other more subtle presentations can be greatly helped by such analysis.

Partial immunophenotypic analysis was obtained for an 8-year-old Golden Retriever with 5100 lymphocytes/μL (reference interval 1000–4800 cells/μL) during a routine recheck for mast cell neoplasia (Figure 2). No abnormalities in lymphocyte morphology were noted. However, the dog had an increased percentage of CD8+ T cells (42%), and 13% of CD3+ T cells had lost expression of CD45. PARR clonality assessment indicated this patient had a clonal population of T cells. Together these findings strongly suggested a diagnosis of T-cell CLL, but because the dog was asymptomatic, it was monitored without treatment. Cases such as this emphasize the importance of studies that include clinical follow-up and multiple immunophenotypic and molecular assessments.

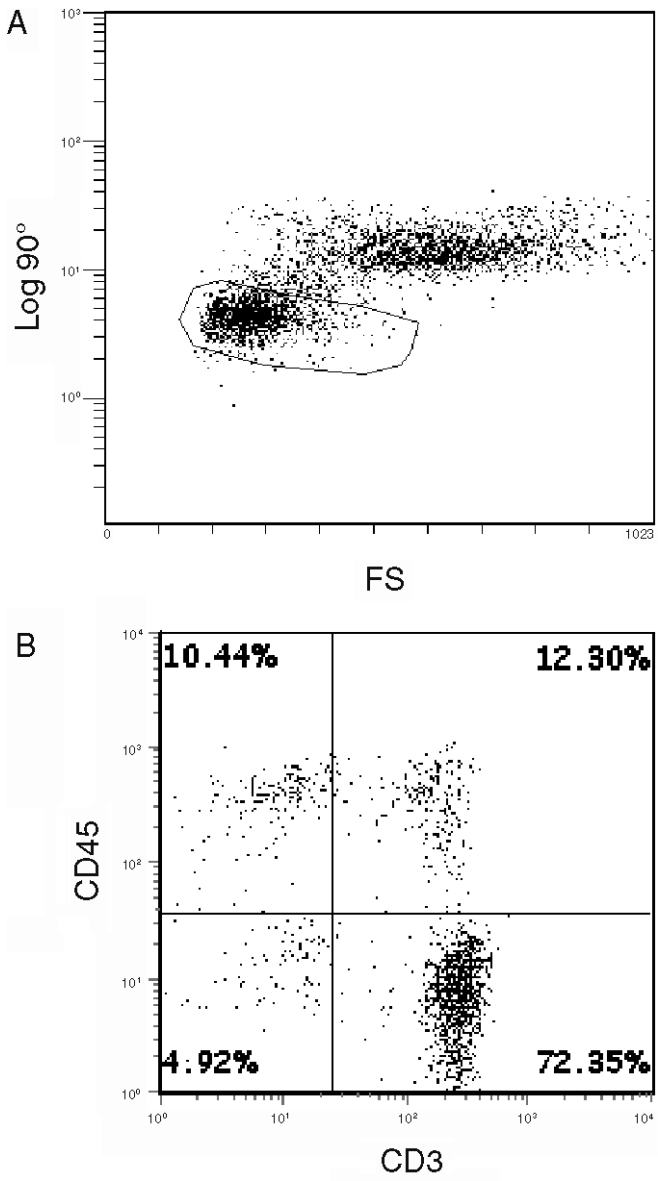

Figure 1. Aberrant antigen expression in a case of canine chronic lymphocytic leukemia demonstrated by flow cytometry. (**A**) Forward (FS) and 90° scatter plot showing the lymphocyte gate. (**B**) Lymphocytes stained with anti-CD3 (T-cell antigen) and anti-CD45 (a pan-leukocyte antigen). A majority of CD3+ T cells have lost expression of CD45. Quadrants were drawn based on isotype controls.

Determination of clonality

In human medicine, routine diagnosis of lymphoid malignancies includes cytologic and histologic examination of cell morphology and immunophenotyping. Determination of clonality—either by testing for homogeneity in immunoglobulin (Ig) light-chain expression or by detecting clonally rearranged antigen-receptor genes—are the tests of choice if routine cytology, histology, or immunophenotyping are unable to provide a definitive diagnosis of malignancy.[27,28] As with any kind of diagnostic testing, clonality assays can give both false positive and false negative results and must

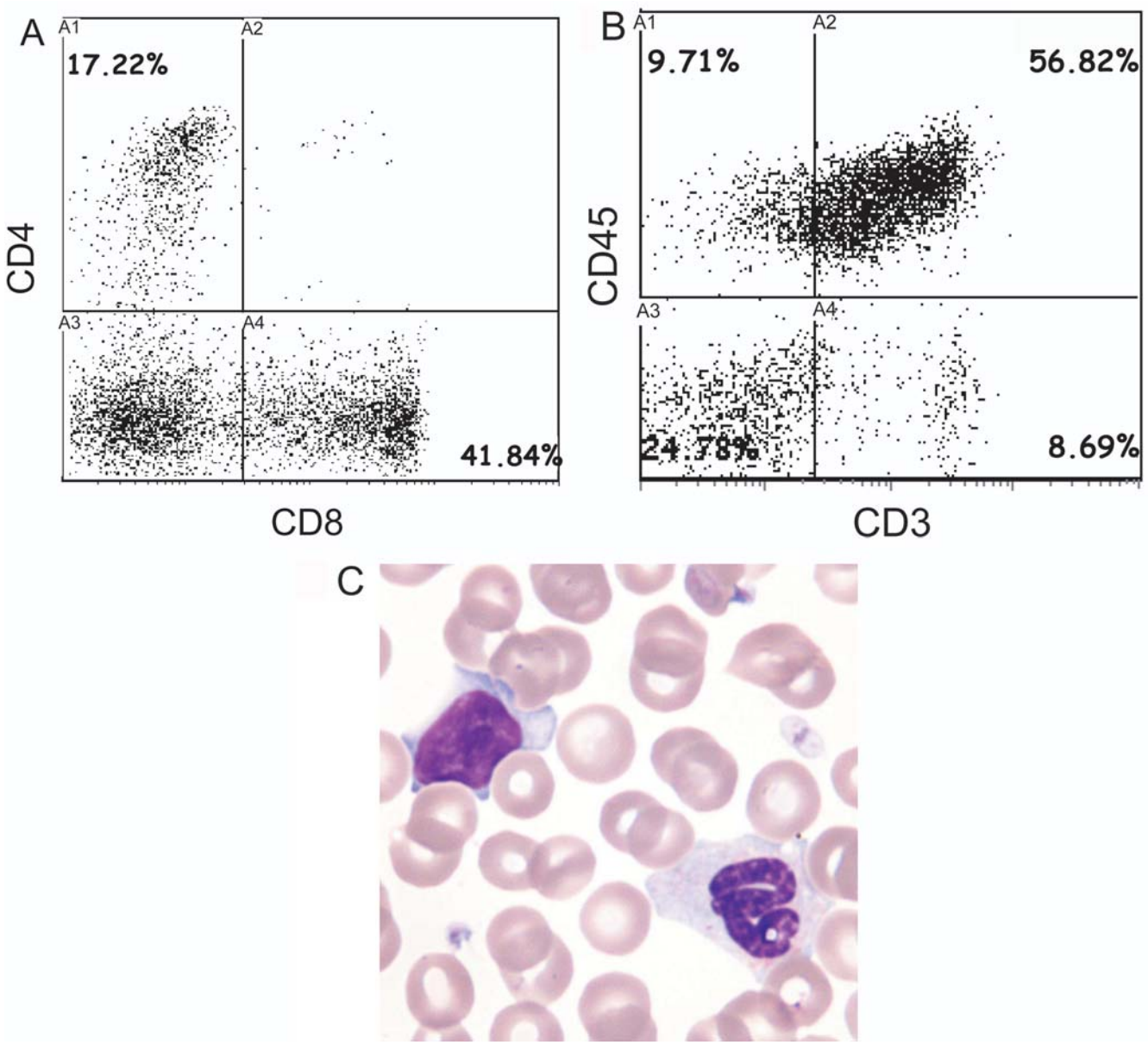

Figure 2. Detection of a small number of aberrant T cells in the diagnosis of leukemia in a dog. (**A**) Lymphocyte expression of CD8 and CD4 showing an increased percentage of CD8+ T cells. (**B**) CD3 and CD45 expression by the same cells as in **A.** A minority of T cells has lost expression of CD45. (**C**) Peripheral blood lymphocyte and neutrophil from this patient. Wright-Giemsa, ×100 objective.

be interpreted with knowledge of the sensitivity and specificity of the assay and the other diagnostic and clinical findings.[28]

Clonality testing is based on the observation that lymphocytes mount a diverse response to antigens, whether they are derived from the environment (eg, allergens), from pathogens, or from self (autoantigens). By contrast, malignant lymphocytes are homogeneous, arising from a single transformed cell. Normal lymphocyte differentiation depends on the rearrangement of genes encoding lg antigen-receptor genes in B cells and T-cell receptor genes in T cells.[29] During this process (Figure 3), nucleotides are trimmed or added between genes as they recombine, resulting in significant length and sequence heterogeneity within the complementarity-determining region 3 (CDR3). Further diversity within B-cell Ig genes is created by somatic hypermutation during antigen-driven B-cell activation. The end result of this differentiation is a diverse population of lymphocytes with virtually limitless antigen specificity and a large variety of CDR3 sequences and lengths.

Lymphocytes derived from the same cell will have CDR3 regions of the same length and sequence. To establish that lymphocytes within a single population have a CDR3 of uniform length, DNA from the cells of interest is amplified by PCR with primers directed at conserved regions of the V and J genes (schematically depicted in Figure 3). The amplified DNA then is separated by size using one of a variety of methods, including polyacrylamide gel electrophoresis followed by ethidium bromide staining, and GeneScan (Applied Biosystems, Foster City, CA, USA) for analysis of fluorescently labeled PCR products. The presence of a dominant single-sized product indicates the presence of a group of lymphocytes that share an identically sized CDR3 region, ie, a clonal expansion of lymphocytes. The presence of many-sized products suggests a polyclonal population of lymphocytes. Lymphoid malignancies also can exhibit biclonal rearrangements, which often are attributed to nonproductive rearrangements on the second chromosome. In some cases, lymphoid malignancies can exhibit an oligoclonal pattern of gene rearrangements,[30] suggesting that the neoplasm has arisen from multiple transformed clones or that individual progeny of the original transformed clone have undergone subsequent CDR3 rearrangement.

An alternative method of determining clonality is to demonstrate both size and sequence homogeneity in PCR products using methods such as heteroduplex analysis and melt-curve analysis. The principle of melt-curve analysis is that a double-stranded PCR product will melt at a discreet temperature that is dependent on its nucleotide content. In a heterogeneous sample, a PCR product of multiple different sequences will melt over a range of temperatures, whereas when the majority of a PCR product is a single species, it will melt at a single temperature. This method has been validated for detecting lymphoma in human[31] as well as canine patients (Burnett et al, manuscript in preparation). Such an analysis has theoretical advantages over size separation of PCR products because similarly sized products with different nucleotide sequences can be distinguished, suggesting that the method may result in fewer false-positive results. This has not yet been tested experimentally, however.

As mentioned above, clonality testing is the most important adjunctive diagnostic test for cases of human lymphoid malignancy. Estimates in human studies suggest a sensitivity of 70–90%, depending on the primers used.[32–35] A few studies address the specificity of identifying a clonal population in people with benign cytologic or histologic findings and suggest a false-positive rate of approximately 5%.[34,35] One of the difficulties with most studies of false positive results in humans is that this designation usually is based on negative cytology or histology findings alone and not on the eventual clinical course. As discussed below (transitional malignant states), detection of malignant clonally expanded lymphocyte populations by molecular methods can precede a cytologic or histologic diagnosis of neoplasia by months or even years.

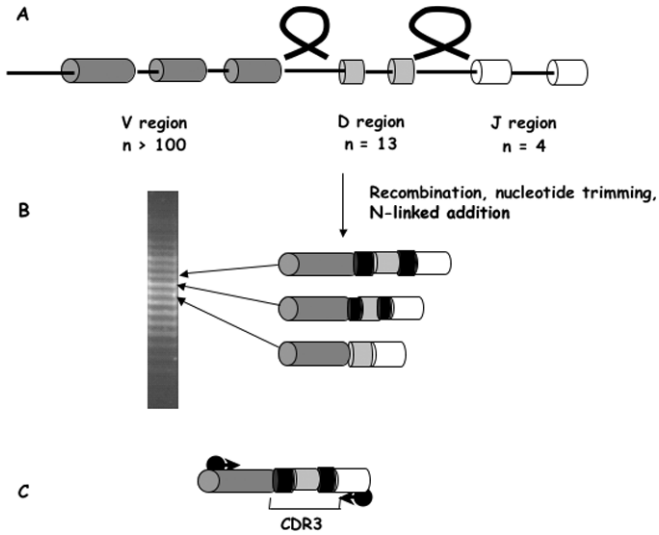

Figure 3. Immunoglobulin gene recombination in lymphocyte differentiation. (**A**) One V-region gene (of more than 100 in the human genome) recombines with a randomly selected D-region gene and a J-region gene, looping out the intervening DNA. (**B**) During this process, nucleotides are added (black bars) between genes, which generates length and sequence diversity in the CDR3. (**C**) Primers with homology to the conserved framework regions of V and J (arrows) will amplify PCR products of different sizes when the DNA is derived from different lymphocytes. Primers are located outside the hypervariable CDR3 region. Differentially sized DNA can be separated on a polyacrylamide gel and will appear as a ladder representing different populations of B cells (**B**, left). Similar principles apply to T cell receptor gene rearrangement.

There are a few studies on human patients that address false-positive results using extended clinical follow-up. In one study, 80 cases of atypical or benign lymphoid hyperplasia were tested for clonality, and in all cases where a clonal population of T or B cells was detected (n = 5), the patients went on to develop malignancy within 2 years.[36] In a separate study, 81% of patients with clonal lymphocyte populations, but no histologically- or cytologically-confirmed malignancy, went on to develop lymphoma during the subsequent year.[37] These data, together with our increased understanding of how malignancies develop, suggest that clonality analysis can provide powerful insights into early disease states.

Clonality testing for dogs now is available on a routine basis. The first large-scale study of this technique was reported by Burnett et al[38] following earlier studies that demonstrated the presence of clonally rearranged T-cell receptor genes in canine

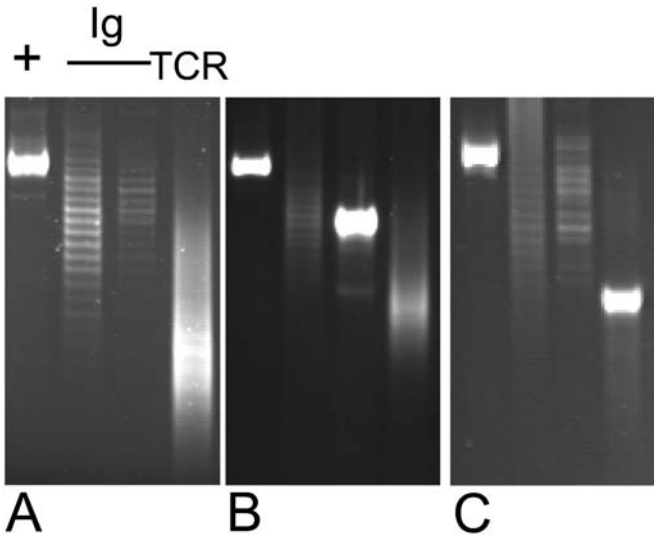

Figure 4. Uniform size of PCR products as an indicator of clonality. Each panel shows 4 PCR reactions on a single DNA sample. The first lane (left) in each panel is a positive control indicating that DNA is present (any nonrearranged gene would be an appropriate target for this reaction). The middle 2 lanes represent 2 different reactions amplifying immunoglobulin CDR3, and the last lane shows TCRγ CDR3 amplification. Samples are separated on a polyacrylamide gel. (**A**) Lymph node aspirate from a normal dog. (**B**) Lymph node aspirate from a dog with histologically confirmed, multicentric B-cell lymphoma. (**C**) Lymph node aspirate from a dog with histologically confirmed T-cell lymphoma.

malignancy (Figure 4).[7,39,40] The assay detects clonal rearrangements in 85% of confirmed lymphoid malignancies (lymphomas, leukemias, and myelomas)[41] (and Burnett et al, unpublished results).

Lack of a clonally rearranged antigen-receptor in a confirmed case of lymphoma or leukemia can have several explanations. First, and most likely, the malignancy may use a V- or J-region gene segment that does not hybridize to the primers used. Diversity of V- and J-region Ig and T-cell receptor gene segments in dogs soon will be clarified by determination of the complete canine genome, which will help establish the degree to which current primers cover these gene segments. Second, the neoplastic lymphocytes may be natural killer (NK) cells, which have unrearranged antigen-receptor gene segments. NK cell tumors appear to be relatively rare, and currently a diagnosis is made by a process of exclusion because NK-specific markers are not commercially available for dogs or cats. Lymphoid malignancies derived from early precursors or with only partially rearranged antigen-receptor gene segments also will be negative with clonality assays. Finally, the number of malignant cells may be below the limit of detection, which is between 1:100 and 1:1000 malignant cells within a background of normal lymphocytes.[33,38] Primer sets have also been developed for feline Ig and T-cell receptor gene rearrangements, but their use is less well-developed (Burnett and Avery, unpublished observations). At present, the primers used in our laboratory can detect approximately 60% of confirmed feline lymphomas and leukemias, and we are working toward developing better reagents.

In a case series of 72 dogs with conditions other than lymphoid malignancy, we found a false-positive rate of 8% for the clonality assay.[41] Leukemias with a suspected or confirmed myeloid origin were excluded from this case series because in people[42] and dogs,[38] acute myelogenous leukemias can have aberrantly rearranged lymphocyte antigen-receptor genes.

False-positive clonality results can have several explanations. First, rare cases of infectious disease can give rise to a clonal population of lymphocytes, although the mechanisms behind this phenomenon are unclear. We[38] and others[7] have reported on 2 dogs infected with *E canis* that had a clonal T-cell population. At least 1 of these dogs was alive and healthy 2 years following the positive result, making occult malignancy unlikely (no follow-up clonality assay was carried out). One dog with Lyme disease was positive for a clonal B-cell population in both peripheral blood and cerebrospinal fluid. The lymphocyte population in the blood disappeared after treatment, and the dog remained healthy 8 months later (Burnett and Avery, unpublished observation). Thus in rare instances, infectious disease can stimulate a vigorous response by a single clone or a small number of clones with same-sized CDR3 regions. The reason for these restricted responses to antigenically complex organisms is not clear.

A second reason for a false-positive clonality result is that the tissue being tested has few resident lymphocytes (eg, nonlymphoid tissue) or poor quality DNA (eg, formalin-fixed samples). In these cases, DNA may be amplifiable from only a small number of normal lymphocytes and thus may give the impression that the tissue contains a clonal lymphocyte population.[43] This phenomenon has been called pseudoclonality. For this reason, it is important to develop sensitivity and specificity values for assays performed on different types of tissue (particularly formalin-fixed versus fresh).

Clonality of B-cell malignancies can also be demonstrated by restricted light-chain usage. Several reports suggest that canine plasma cell tumors are mostly[44] or exclusively[45] λ–light-chain positive; uniform λ-positivity and negative staining with κ-chain antibodies was used to establish that these tumors were clonal. The fact that 91% of canine and feline B cells express Igλ[46] suggests that this technique should be used with caution, however, because even heteroge-

neous B-cell expansions will be predominantly Igλ-positive.

Chromosomal abnormalities

Lymphomas and leukemias frequently are associated with translocations because the process of recombining antigen-receptor genes leaves lymphocytes susceptible to mistakes in recombination. Most translocations found in human leukemias and lymphomas involve the Ig heavy-chain gene locus. For example, the t(11;14) translocation juxtaposes the locus encoding cyclin D1 on chromosome 11 to an Ig-enhancer sequence on chromosome 14. This translocation, which results in the overexpression of cyclin D1, is found in virtually all cases of mantle-cell lymphoma.[47] Detection of the translocation by PCR[47] or of the overexpressed protein by immunohistochemistry[48] can be used to confirm a diagnosis of mantle-cell lymphoma in histologically ambiguous cases. Recently, a consortium of European researchers found that the combined use of clonality determination through antigen-receptor rearrangements together with detection of this and other translocations by PCR resulted in detection of a clonal population in 95% of cases with confirmed lymphoid malignancies.[49]

Chromosomal aberrations have been detected in dogs by conventional karyotyping[50] and by comparative genome hybridization.[6] Aberrations include the gain or loss of portions of chromosomes as well as balanced translocations between different chromosomes. The assays used to detect chromosomal changes are not yet amenable to routine diagnostic testing and may not be sufficiently sensitive for detecting early malignancy or a small number of malignant cells within a population of reactive cells. Despite this, studies will almost certainly lead to the discovery of targeted PCR and immunohistochemistry or flow cytometry-based assays that can be used for detecting malignant lymphocytes in ambiguous cases.

Gene expression profiling

The ability to analyze the expression levels of thousands of genes simultaneously through gene expression profiling has had immense scientific and clinical benefits in the study of human leukemias and lymphomas. For example, human CLL is a B-cell disease with a consistent immunophenotype (CD5+ and CD21+) but a heterogeneous clinical picture. Some patients experience prolonged survival without treatment, whereas other patients develop a more rapid clinical course in the face of therapy. Studies of the hypervariable region of the Ig genes expressed by CLL B cells found that they fall into 2 categories with striking prognostic significance: leukemias with hypermutated Ig genes, which indicates they have encountered antigens and divided within germinal centers, and leukemias with germline or unmutated Ig genes.[51] Patients with leukemias that had no somatic hypermutation had a mean survival time of 8 years after diagnosis, whereas patients with leukemias that had hypermutated Ig genes had a mean survival time of 24 years. Sequencing the hypervariable region is not yet feasible as a routine diagnostic tool, so oncologists sought additional prognostic indicators. Rosenwald et al and Wiestner et al[52,53] used gene expression profiling to sift through thousands of genes expressed by leukemias with a good prognosis and leukemias with a poor prognosis and found that expression of the tyrosine kinase ZAP-70 is highly correlated with clinical outcome. Subsequently, this same group developed a flow cytometry-based diagnostic test for this protein that can be readily put to routine clinical use.[54] This series of studies, which spanned only a few years, demonstrates the power of gene expression profiling to derive clinically useful information in a relatively short period of time. Gene expression profiling of dogs is possible using commercially available Affymetrix chips (Santa Clara, CA, USA), and it is likely that studies similar to those described above will yield useful diagnostic and prognostic markers in this species.

Transitional Neoplastic States

The increased use of many of the tools described above, particularly clonality assessment, has increased the ability of pathologists to detect early and occult malignancy, with some surprising findings. In particular, there is a growing list of human inflammatory and infectious diseases associated with an increased likelihood of transition from reactive to malignant lymphocyte expansion. These transitional states provide particular diagnostic dilemmas because relying solely on the typical morphologic criteria for lymphoma and leukemia can result in delayed or incorrect diagnoses. Delayed recognition of these transitional states can be quite costly because early intervention to remove the antigenic stimulus can halt the progression toward incurable disease. Conclusive evidence for these transitional neoplastic states in small animal medicine currently is lacking, but intriguing correlates with human disease exist and warrant further investigation.

Mucosa-associated lymphoid tissue lymphoma induced by *Helicobacter pylori*

H pylori is a gram-negative, spiral bacterium that is commonly found as a component of the gastrointestinal (GI) flora of people. In the early 1990s, chronic infection with *H pylori* was shown to be associated with GI tumors, including mucosa-associated lymphoid tissue (MALT) lymphoma (a subtype of low-grade B-cell lymphoma most commonly found in the stomach).[55] Remarkably, eradication of *H pylori* with antibiotics resulted in regression of the gastric MALT lymphoma in approximately 80% of patients.[56,57] Histologic regression can take up to a year to occur, and when patients are followed for several years, relapse rates are generally less than 10%.[56] MALT lymphomas that do not respond to antimicrobial therapy often have chromosomal translocations or are detected at an advanced stage.[58]

T lymphocytes reacting to *H pylori* release growth factors contributing to an initial expansion of B lymphocytes.[59,60] This antigen-driven expansion of B lymphocytes appears to set the stage for subsequent transforming events that culminate in the emergence of a clonally-derived tumor. A direct pathogenic link between *Helicobacter* infection and MALT lymphoma was established by demonstrating that the eventual malignant clone could be found in inflammatory lesions years before the development of histologically identifiable lymphoma.[61] Thus, the identification of an early malignant or premalignant state using sensitive detection methods can have an impact on patient management and clinical outcome.

H pylori infection in animals

Several naturally occurring and experimental animal models for *Helicobacter*-induced gastric inflammation have been described, some of which mimic the early lymphoid response in *H pylori*-infected people. New *Helicobacter* species have been described in association with gastritis in several species, including ferrets, cats, cheetahs, and swine.[62] Not only do cats naturally harbor *Helicobacter felis* and other related species, but they can be infected with *H pylori* in laboratory and commercial breeding settings.[63] *H felis* and *H pylori* infections in cats can lead to dramatic lymphoid follicular hyperplasia similar to that seen in people in response to *H pylori*.[64,65]

Immunophenotyping in cats with *Helicobacter*-associated lymphoid hyperplasia has shown the proliferating lymphocytes are primarily B cells[64]; in some cases, the B cells have downregulated an epitope normally associated with apoptosis.[65] Thus, there is evidence for early, potentially unregulated, B-cell hyperplasia in feline *Helicobacter* infections. *H felis* infection commonly occurs in dogs as well, and in experimental infections can result in lymphoid hyperplasia. Mice infected with *H felis* have developed gastric MALT lymphoma.[66] Although *Helicobacter* infection of dogs and cats results in the early lymphocytic immune response seen in people, a link to subsequent gastric lymphoma remains to be established.

Celiac disease in people

Celiac disease is a well-studied human enteropathy that recently was linked directly to the development of intestinal T-cell lymphoma.[67–69] Celiac disease is characterized by chronic diarrhea and weight loss due to malabsorption. The disease is the result of an abnormal T-cell response to gluten and gluten-like molecules in wheat and other grains (reviewed by Greenberger and Isselbacher[67] and Schuppan[69]).

Histologically, celiac disease is characterized by villous blunting and infiltration of lymphocytes (primarily T cells) in the lamina propria and the epithelium. Uncomplicated celiac disease responds to withdrawal of gluten from the diet. However, a subset of 5 to 10% of patients are refractory to gluten withdrawal.[68] Small clinical studies have demonstrated that a substantial percentage of patients with refractory celiac disease develop intestinal T-cell lymphoma.[70–72] Even in the absence of overt histologic T-cell lymphoma, patients with clonal, intestinal T-cell populations suffer a malignant disease course, often dying of malnutrition.[72] This has lead to the proposition that refractory celiac disease with clonality should be considered a cryptic T-cell lymphoma that over time would ultimately progress to overt lymphoma. Neoplastic T cells can be identified both by clonality studies and by aberrant antigen expression,[19] further illustrating the utility of sensitive techniques for the diagnosis of early malignant states.

Feline inflammatory bowel disease

The GI tract is a common site for both lymphocytic-plasmacytic inflammation[73] and lymphoma[74,75] in cats, and drawing a clear line between the 2 conditions can be problematic. Feline lymphocytic-plasmacytic enteritis is a histologic diagnosis defined by an excess of lymphocytes and plasma cells in the lamina propria, and which may be accompanied by blunting and thickening of the intestinal villi. Recent reports of feline intestinal lymphoma have documented a high proportion with T-cell phenotype[76] and a low-grade (lymphocytic),

epitheliotropic histologic appearance.[77] Carreras et al[77] report on a subset of chemotherapy-responsive cats with epitheliotropic, T-cell lymphoma with survival times ranging from 23–29 months. Intriguingly, 1 cat treated with prednisone and a novel protein diet was alive 28 months after the initial diagnosis. It is tempting, when considering the frequency of both reactive and neoplastic intestinal lymphocyte expansions in cats, to speculate that feline intestinal lymphoma is a model of antigen-driven lymphocyte expansion with subsequent transformation. Whether dietary antigens or GI pathogens play a role in T-cell tumors will require further studies using molecular techniques to investigate the inflammatory stages of disease.

Canine ehrlichiosis

Canine *E canis* infection is characterized by profound changes in the immune system, including destruction of platelets,[78,79] monoclonal gammopathy,[80] and dramatic expansion of LGLs.[2–4] In one study, a kennel of chronically infected dogs developed persistent lymphocytosis, which in several cases exceeded 10,000 cells/μL.[12] Dogs infected with *E canis* also appear to develop, on occasion, clonally expanded T-cell populations. Vernau et al[7] reported 1 such dog, and in the previously described population of dogs, *E canis* infection was one reason for the finding of a clonal T-cell population in a dog without overt lymphoid malignancy. It appears that a subset of dogs with *E canis* infections develop T-cell expansion, monoclonal gammopathy, and lymphocytosis, consistent with chronic antigen-driven lymphocyte activation. As described above, such conditions can ultimately progress to malignant transformation. Further study will be required to determine if untreated canine ehrlichiosis represents such a transitional disease state.

Emerging antigen-driven immunoproliferative states

Additional associations between infectious organisms and lymphoma have begun to emerge in people. *Tropheryma whipplei* is a gram-positive bacillus found in soil. Infection with the organism can lead to Whipple's disease, a multisystemic infection resulting in diarrhea, abdominal pain, weight loss, lymphadenopathy, and fever. B-cell lymphoma has been reported rarely in association with Whipple's disease, and recently, resolution of a monoclonal B-cell proliferation in response to antimicrobial therapy against *T whipplei* was documented.[81]

Hepatitis C virus (HCV) infection has been proposed as a potential initiator of some non-Hodgkin's lymphomas.[82] In a recent report, complete, durable clinical remission was observed in 8 of 9 patients diagnosed with splenic lymphoma with villous lymphocytes and treated with the antiviral cytokine interferon-α.[83] Remission was associated with loss of detectable HCV virus RNA, and in 1 patient, relapse was temporally associated with a return of detectable HCV RNA. Induction of B-cell proliferation does not appear to be due to direct infection with HCV and may represent another example of antigen-driven lymphocyte expansion. It is likely that, with closer examination of different types of lymphoma, the list of microorganisms with the potential to initiate the transition from antigen-driven lymphocyte expansion to neoplasia will continue to grow.

Conclusions

As our understanding of lymphocyte transformation grows, it has become increasingly apparent that more sensitive methods for detecting lymphoma are needed. Widespread use of such emerging techniques will likely change the way we view lymphoid malignancies in veterinary medicine and may lead to earlier and more efficacious treatment strategies. Whether the goal is increasing the sensitivity and specificity of the initial diagnosis, linking antigen-driven proliferative states to increased risk of lymphoma, or improving the efficacy of current chemotherapy protocols, molecular detection methods soon will become essential tools for veterinary pathologists.

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