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Veterinary Immunology and Immunopathology xxx (2005) xxx–xxx

**Veterinary
immunology
and
immunopathology**

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Lineage differentiation of canine lymphoma/leukemias and aberrant expression of CD molecules

M.J. Wilkerson^{a,*}, K. Dolce^a, T. Koopman^a, W. Shuman^a,
R. Chun^b, L. Garrett^b, L. Barber^c, A. Avery^d

^a Department of Diagnostic Medicine/Pathobiology, Kansas State University,
1800 Denison Avenue, Manhattan, KS 66506, USA

^b Department of Clinical Sciences, Kansas State University, USA

^c Department of Clinical Sciences, Tufts School of Veterinary Medicine, USA

^d Department of Pathology, Colorado State University, USA

Received 7 September 2004; received in revised form 26 January 2005; accepted 4 February 2005

Abstract

Multiparameter flow cytometry analysis and specific cluster differentiation (CD) molecules were used to determine the expression profiles of B- and T-cell antigens on lymph node preparations from 59 dogs with generalized or multisystemic lymphoma. Lymph node samples from 11 healthy dogs were labeled to validate the specificity of antibodies and to formulate guidelines for interpretation of the results obtained from lymphoma samples. In normal lymph nodes, T-lymphocytes expressing CD3, CD4, or CD8 β represented $59 \pm 11\%$, $43 \pm 8\%$, or $16 \pm 5\%$ of the total cells, whereas B-lymphocytes expressing either CD21 or surface IgM (IgM) represented $37 \pm 9\%$ or $14 \pm 5\%$, respectively. Small lymphocytes could be distinguished from large lymphocytes by forward light scatter. Of the patient samples 29 different breeds were represented with Golden and Labrador retriever being the most common. The lymphoma samples segregated into three groups based on CD antigen expression. Thirty cases predominantly expressed one or more combinations of CD79a, IgM, and CD21 representing a B-cell lineage. Three B-cell cases also expressed the stem cell antigen, CD34. Sixteen cases expressed one or more combinations of CD3, CD4, and CD8 consistent with a T-cell lineage and CD3+CD4+CD8– phenotype was the most common. Thirteen cases showed a mixed expression profile for T- and B-cell antigens and in three cases CD14 was highly expressed. Clinical response was poorest for T-cell lymphomas. Leukemic states occurred in all three phenotypes; but mixed cell cases had the greatest proportion. Dual immunofluorescence staining confirmed co-expression of T-cell (CD3) and B-cell antigens (CD79a or CD21) on neoplastic lymphocytes of six mixed cell cases. In one mixed cell case, dual immunostaining identified lymphocyte populations that stained mutually exclusive for CD79a and CD3. Six mixed cell lymphomas tested by PCR showed clonality for rearranged antigen receptor. Four cases that were CD79a+CD3+ had TCR γ chain gene rearrangements, whereas two cases that were CD3+CD8+CD21+ had Ig heavy chain rearrangement. One case expressing multiple CD molecules (CD3+CD8+CD21+CD14+) was PCR negative for both Ig and TCR γ gene rearrangement and could not be classified into a B- or T-cell lineage. We show for the first time co-expression of B- and T-cell markers on lymphoma cells that had specific T- or

* Corresponding author. Tel.: +1 785 532 4818; fax: +1 785 532 4072.

E-mail address: wilkerns@vet.ksu.edu (M.J. Wilkerson).

B-cell gene rearrangements. These findings suggest that aberrant CD molecule expression is not an uncommon finding in canine lymphomas and is a useful diagnostic marker for malignancy.

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Keywords: Cluster of differentiation; Dog; Flow cytometric analysis; Lymphocyte phenotyping; Lymphomas; PCR

1. Introduction

Canine lymphoma is a common, spontaneously occurring hematopoietic tumor in dogs and a model for non-Hodgkin's lymphoma in humans (Teske, 1994a). World Health Organization (WHO) classifications for human and canine lymphoma/leukemias are similar, but the prognostic value of the classification system in dogs needs further studies (Raskin, 2004b). The significance of the immunophenotype of lymphocytes as a prognostic factor has been consistently confirmed and T-cell lymphoma has a poorer prognosis than B-cell lymphoma (Dobson et al., 2001; Ruslander et al., 1997; Teske et al., 1994). Two factors that consistently correlate with prognosis in dogs are immunophenotype and WHO substage status (Greenlee et al., 1990; Keller et al., 1993; Teske, 1994b; Valli et al., 2002; Raskin, 2004a). Recently, two studies showed that dogs with aggressive lymphoproliferative disease as depicted by short survival times were not all T-cell, but included some diffuse large and Burkitt-type B-cell subtypes (Raskin, 2004a) indicating that phenotype is not the only important prognostic contributor (Ponce et al., 2004). Immunophenotyping can be performed by flow cytometry and by immunohistochemistry. The advantage of immunophenotyping by flow cytometry is that smaller samples obtained by less invasive techniques can be analyzed. In contrast, immunohistochemistry has the advantage over flow cytometry methods in that identification of the cell subsets can be made in the context of the lymph node architecture. Ideally assessment of both abnormal T- and B-populations should be made in tissue sections; however, this is not easily accomplished due to alterations in the B- and T-specific epitopes that may occur after tissue fixation and tissue processing steps. With the advent of fluorochrome conjugated canine monoclonal antibodies, multi-color immunofluorescent labeling can be performed by flow cytometry to detect cells expres-

sing multiple cluster differentiation (CD) molecules simultaneously.

Lineage differentiation of neoplastic and reserve (normal) lymphocyte populations can be determined by the expression of CD molecules using a broad panel of markers and light scatter properties of the cells analyzed by flow cytometry. Noncommitted hematopoietic stem cells express CD34, a glycosylated surface glycoprotein. This marker is often used to differentiate acute immature leukemias of lymphoid or myelogenous origin from chronic lymphocytic leukemia or a leukemic state of lymphoma (Moore and Vernau, 2004). B-cell lineages are typically defined by the expression of cytoplasmic CD79a and other CD molecules to determine the maturation state of the lymphoma cells. Stages of B-cell development can be identified by the sequence of cell marker expression. Early pre-B-cells express cytoplasmic CD79a and μ (IgM) as the B-cell prepares for antigen receptor gene rearrangement of the heavy and light chains (Cooper, 1987). CD79a, the signal transduction portion of the B-cell receptor spans the transmembrane and intracytoplasmic regions of the cell and is expressed in all immature and mature B-cells and in the majority of B-cell neoplasms, including precursor B-cells and plasma cell tumors (Chu and Arber, 2001). The pre-B-cells become immature B-cells when cytoplasmic IgM is exported to the surface. As immature B-cells naïve to antigen exposure exit the bone marrow, CD21 and IgM appear on the surface (Moore and Vernau, 2004; Aster and Kumar, 1999).

Cells that enter the thymus are capable of giving rise to T-cell subsets, NK cells, and dendritic cells. Markers that are present on early T-cell precursors (thymocytes) include cytoplasmic CD3, CD2, CD5, CD7, and CD1a (Strominger, 1989). CD3 is a complex molecule associated with the T-cell receptor (TCR) and is expressed in early thymocytes and throughout maturation. During thymic maturation, the T-cell precursors undergo rearrangement of the TCR and

begin to express the accessory molecules CD5, CD4, and/or CD8. Mature T-cells that enter the circulation and lymph nodes lose early thymic markers (CD1a) while retaining surface CD3, CD4 or CD8 (Moore and Vernau, 2004; Rothenberg et al., 2003).

In humans identification of B-cell clonality can be identified when neoplastic cells exclusively express kappa or lambda light chains. This strategy is more difficult to apply in canine samples because lambda light chain expression predominates over kappa light chain in normal dog lymphocytes (Arun et al., 1996). To date there are no specific cell markers that define T-cell clonality in any species. Therefore, identification of B- or T-cell clonality in dogs requires molecular studies for receptor gene rearrangement (Burnett et al., 2003). During development and early maturation lymphoid cells undergo genetic rearrangements of genes encoding the immunoglobulin (Ig) and the T-cell receptor (TCR) that lead to sequential expression of the receptors of B- and T-lineage. In most lymphoid neoplasias, antigen receptor gene rearrangement precedes transformation; therefore, the daughter cells derived from the malignant progenitor have the same antigen receptor gene, representing a monoclonal population. Using PCR assays that identify genes that encode for T- and B-cell antigen receptors, it is possible to differentiate monoclonal neoplasms from polyclonal reactive processes and to screen for minimal residual disease after treatment or recurrences before they become clinically apparent.

In this study, we illustrate how CD molecule expression and forward angle light scatter properties can be used to distinguish abnormal from normal lymphocyte populations in lymph node samples obtained from canine patients with lymphoma/leukemia previously diagnosed by cytology or histopathology. We show for the first time lymphoma cases that co-express various combinations of T- and B-cell CD markers using dual-fluorochrome color analysis. Lineage classifications into T- or B-cell phenotype and clonality of these unusual cases were confirmed by receptor gene rearrangements using published molecular methods indicating that aberrant expression of CD molecules is a characteristic of canine lymphoma, and as in human lymphoproliferative diseases, is strong evidence for a diagnosis of a lymphoid malignancy.

2. Materials and methods

2.1. Case accession

Cases of lymphoma (59) were submitted from the oncology services of Tufts School of Veterinary Medicine (Tufts), North Grafton, Massachusetts, and Kansas State University (KSU) Veterinary Teaching Hospital, Manhattan, Kansas. Complete histories, signalments, and clinical assessments were collected on all dogs. All dogs included in the study met the WHO stage 3 or greater, substages a or b. Staging consisted of a database which included a complete blood count (CBC), serum biochemistry, urinalysis, thoracic and abdominal radiographs, bone marrow aspirate, and lymph node aspirate with or without biopsy. The diagnosis of lymphoma was based on the cytologic or histopathologic assessment of single or multiple lymph node samples obtained by needle or trucut biopsies. The response to induction therapy (modified University of Madison, Wisconsin used at KSU or VELCAP-RT used at Tufts) was categorized by the attending oncologist according to three-dimensional measurements of peripheral lymph nodes. The categories designated included complete remission (CR), a reduction in lymph nodes to normal size and absence of detectable disease persisting for 4 weeks after treatment; partial remission (PR), a greater than 50% reduction in lymph node size persisting for 4 weeks after treatment; no response (NR).

2.2. Immunohistochemistry

Lymph node biopsies obtained from ten cases at Tufts School of Veterinary Medicine were sent to the University of California, Davis for immunohistochemistry using CD79a and CD3 antibodies as described previously (Vernau and Moore, 1999).

2.3. Surface and intracellular immunolabeling and flow cytometry

Popliteal lymph nodes were harvested from 11 healthy dogs, to validate the specificity of the antibody reagents and to develop a reference interval for expression of CD molecules by normal lymphocytes. All procedures performed on animals were approved by the institutional animal care and use committee.

Cellular suspensions from the popliteal lymph nodes were collected using a tissue disassociation kit (Sigma Co., St. Louis, MO).

To obtain tissue for immunophenotyping analysis from oncology patients, multiple needle fenestrations of an affected lymph node was performed, and the tissue was expelled into a red top tube containing 0.5 mL of physiologic saline. To preserve cellular viability during transport from Tufts, samples were placed in RPMI with 2% fetal bovine serum. Samples were sent to the laboratory cooled with ice or cold pack by overnight courier service. All samples were processed within a 24-h period of collection.

Lymph node samples from dogs both healthy and diagnosed with lymphoma, were labeled with a panel of antibodies (Table 1) using a modification of a previously described method (Faldyna et al., 2001). For surface marker labeling, 100 μ L of cells was aliquoted into 10 (12 \times 75) polystyrene tubes (Falcon, Becton Dickinson, San Jose, CA), each containing a separate antibody (10 μ L of antibodies from the University of Davis, CA or 50 μ L at 10 μ g/mL of antibodies from VMRD) and incubated at room temperature for 15 min. Optimal cellular concentration for the labeling procedure was 1×10^7 cells/mL. When short samples were processed, the antibodies were restricted to principally CD45, CD3, CD21 or CD79a, CD14 and CD172a. When necessary, erythrocytes were lysed by adding 3 mL of 0.85% NH_4Cl for 2–5 min, and then centrifuged at $1000 \times g$ for 3 min in a Clay Adams Serofuge (Becton Dickinson,

San Jose, CA) to pellet the cells. The supernatant was decanted and the cells were washed in 3 mL of phosphate buffered saline (0.01 M pH 7.2, PBS) containing 1% gamma globulin free horse serum (Gibco, Life Technologies, Rockwell, MD), and centrifuged as before. Secondary antibody (100 μ L of 10 μ g/mL) of fluorescein-isothiocyanate (FITC) conjugated goat anti-mouse IgG H+L (Bethyl Labs, Montgomery, TX) was added to each tube and incubated at 4 $^\circ\text{C}$ in the dark for 20 min. Following incubation, the cells were washed two times with 3 mL of PBS and resuspended with 250 μ L of PBS. To determine the background fluorescence, one tube of cells was incubated with the FITC-conjugated secondary antibody without the primary antibody. To determine viability of the sample, 10 μ L of propidium iodide at 50 μ g/mL (Becton Dickinson, San Jose, CA) was added to one tube.

B-lymphocytes not expressing CD21 or IgM (i.e. immature or plasma cells) were detected using intracellular CD79a. Two aliquots of cells (100 μ L) were first fixed with 100 μ L of 4% paraformaldehyde in PBS for 30 min and centrifuged. Cells were permeabilized with 0.1% saponin (Sigma Co., St. Louis, MO) in PBS (100 μ L) and simultaneously labeled with either R-phycoerythrin (PE)-labeled CD79a or control PE-IgG1 (10 μ L, DAKO, Carpinteria, CA), then incubated for 20 min at 4 $^\circ\text{C}$ in the dark. Aliquots were washed twice with PBS as described previously and resuspended with 250 μ L of PBS. For dual labeling of a surface marker and

Table 1
Antibody panel used for immunophenotyping canine lymphomas

CD molecule	Specificity	Antibody clone	Source
CD45	All leukocytes	CA12.10C12	UCD (Vernau and Moore, 1999)
CD3	T-lymphocytes	CA17.2A12	UCD (Vernau and Moore, 1999)
CD3-FITC		Same clone	Serotec
CD4	T-lymphocyte subset	CA13.1E4	UCD (Moore et al., 1992)
CD8 β	T-lymphocyte subset	CA15.4G2	UCD (Moore et al., 1992)
CD21	Mature B-lymphocyte	CA2.1D6	UCD (Vernau and Moore, 1999)
B-cell-PE		Same clone	Serotec
Surface IgM (IgM)	Immature B-lymphocytes	Polyclonal, A40-11F	Bethyl Labs
Surface IgG (IgG)	Mature B-lymphocytes	Polyclonal, A40-105F	
CD14	Monocytes	CAM36	VMRD (Brodersen et al., 1998)
CD172a	Granulocytes/monocytes	DH59B	VMRD (Davis et al., 1987)
CD34	Stem cell	1H6	Becton Dickinson (McSweeney et al., 1996; McSweeney et al., 1998)
CD79a-PE	B-lymphocytes, all stages	Polyclonal, HM57	DAKO (Milner et al., 1996)

All antibodies are monoclonal unless otherwise specified in the table.

cytoplasmic CD79a, the cells were first labeled for the surface marker using the antibody for the CD molecule of interest, then processed as described for intracellular staining for CD79a. Additional dual labeling was performed on seven cases using direct surface labeling of cells simultaneously with 10 μ L aliquots of FITC-conjugated CD3 antibody and PE-conjugated CD21 antibody (Serotec, Raleigh, NC). Fluorescent (FITC and PE) conjugated isotype control antibodies (Serotec, Raleigh, NC) were used to set up quadrants for CD21 and CD3 dual labeling.

Labeled cells were analyzed using a FACSCalibur flow cytometer and Cell Quest software (Becton Dickinson, San Jose, CA). Data was collected on 5000 cells gated to exclude dead cells. Cells were collected using: forward angle scatter and side angle scatter in linear mode, FL1 and FL2 in log mode and compensation adjusted as necessary. Cell populations were displayed as scatter plots with FSC-H (size) on the *y*-axis and log fluorescence (FITC or PE) on the *x*-axis. For dual fluorochrome analysis of samples, cellular fluorescence was displayed as scatter plots with PE log fluorescence on the *y*-axis and FITC log fluorescence on the *x*-axis.

Specificity of the antibody panel was verified on lymph node samples harvested from healthy dogs. A typical staining profile from a healthy dog is depicted in Fig. 1. Lymphocytes from healthy lymph nodes separate into two populations based on size. Cells falling between 200 and 400 FSC-H channels represent small cells, whereas cells residing above 400 FSC-H channels represent medium to large cells.

2.4. PCR for antigen gene rearrangements

Molecular methods that determine the presence of clonal gene rearrangements of the B- or T-cell receptor have been previously described and were performed by the clinical immunology laboratory at Colorado State University (Burnett et al., 2003). In this assay, the products of the gene amplicons were electrophoresed in polyacrylamide gel. Control primers were used to detect the μ constant gene region encoding for IgM and loaded into the first lane 1 of each gel. Two primer pairs were used for different junctional regions of the immunoglobulin heavy chain (designated IgH for major and Igh for minor) and a single primer pair for a junctional region of the TCR γ

gene. The amplicons of these sets of primers were loaded in the next lanes in sequence (lanes 2 for IgH, 3 for Igh, and 4 for TCR γ , respectively). Clonality was detected by the presence of a prominent single (monoclonal) band in the gel. A laddering effect in the gel represented amplification of DNA from a polyclonal population of lymphocytes.

3. Results

3.1. CD molecule expression on lymphocytes from healthy dogs

Lymph nodes from 11 healthy dogs were analyzed. The majority of cells in the lymph node preparations expressed CD45 ($92 \pm 5\%$). The degree of CD45 expression varied among lymphocyte populations, where some populations have much more fluorescent intensity than other populations (Fig. 1). T-lymphocytes expressing CD3, CD4, or CD8 β represented $59 \pm 11\%$, $43 \pm 8\%$, or $16 \pm 5\%$ of the cells, respectively. B-lymphocytes expressing CD21 or IgM constituted $37 \pm 9\%$ or $14 \pm 5\%$. Both small (200–400 FSC-H channels) and large lymphocytes (500–800 FSC-H channels) were represented in T- and B-cell populations (Fig. 1). As expected, CD34 ($2 \pm 2\%$) was not expressed on the lymphocyte populations of healthy dogs and CD14+ cells were few ($4 \pm 3\%$). Granulocytes were rare as depicted by CD172a+ cells ($3 \pm 2\%$) and were attributed to neutrophils and eosinophils in cytological and histologic assessment of the lymph node samples.

3.2. Immunophenotype of lymph node samples of oncology patients

A total of 59 cases of lymphoma were analyzed. A total of 29 different breeds were represented, Labrador and Golden Retrievers were the most common breeds affected (12 and 10 out of 59). The dogs in the study ranged from 3 to 14 years of age with an average age of 8.5.

Using the normal lymph node values as a guideline, a cut off of 60% was used to classify patient samples as either B- or T-cell phenotype. Specifically, where a predominance of at least 60% of all cells in the preparation expressed one or more B-cell molecules,

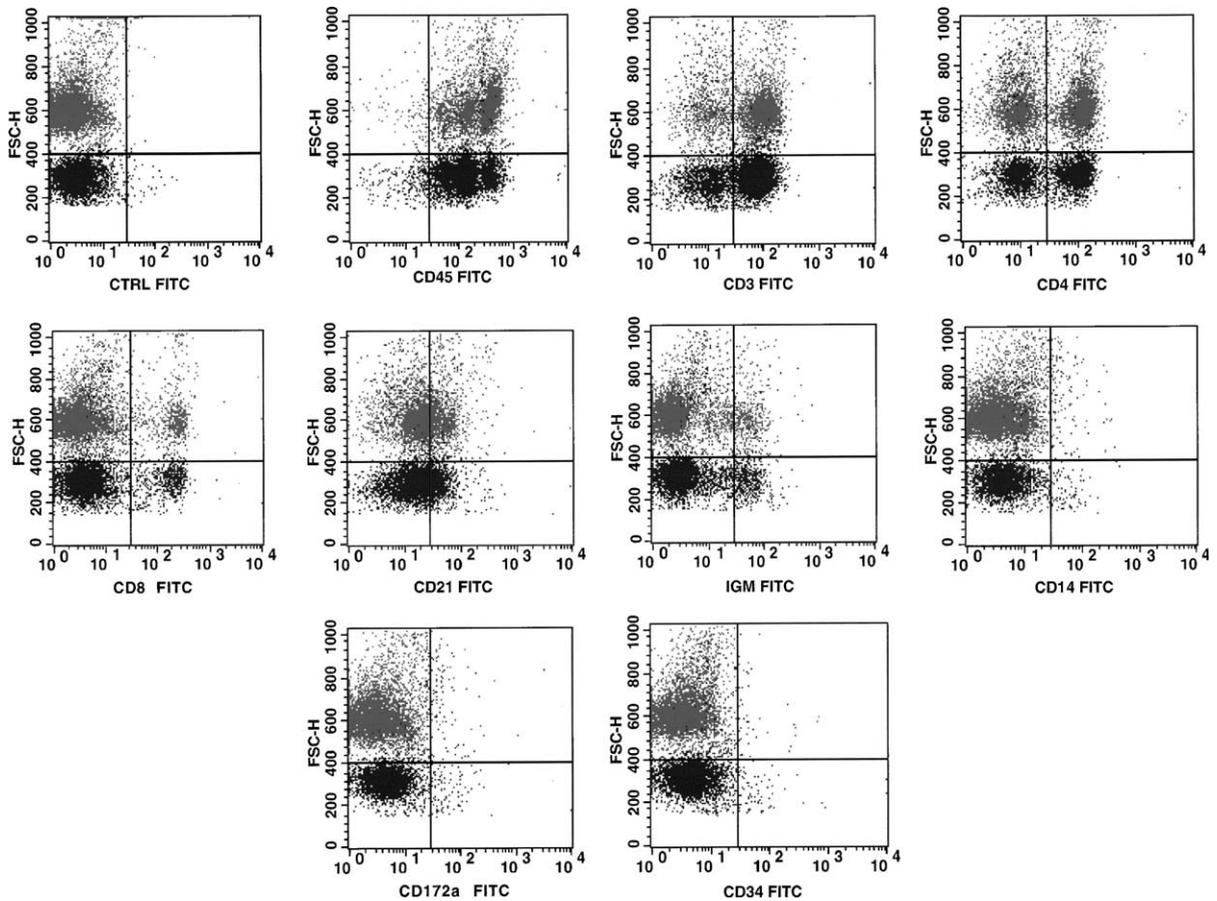


Fig. 1. Flow cytometry plots (FSC-H vs. FITC) of lymph node cells obtained from a healthy dog labeled with fluorochrome-conjugated antibodies to specific cell markers (listed on the *x*-axis). Horizontal line separates small from large lymphocytes based on FSC-H channels (*y*-axis, linear scale). Vertical line is placed to the right of cells labeled with negative control antibody (CTRL FITC, top row, left-hand plot). This line is used to identify populations that expressed CD molecules and shifted to the right in fluorescent intensity (log scale).

the sample was interpreted to be of B-cell lineage. Similarly, if 60% of the population expressed T-cell molecules the sample was interpreted to be of T-cell lineage. Although reduced cellularity precluded the use of a full antibody panel in three cases, one case was determined to be of B-cell lineage due to CD79a expression on 95% of the cells, whereas CD3 was 3% of the total cells and two cases were determined to be of T-cell lineage due to 80–90% expression of CD3 with 10–20% CD21+ cells. Based on these criteria thirty cases predominantly expressed one or more B-cell antigens, and sixteen cases predominantly expressed one or more T-cell antigens. Thirteen cases had immunophenotyping results that were ambiguous suggesting a mixed cell phenotype or aberrant

expression of CD molecules (Table 2). Thirty-three samples were submitted from Tufts School of Veterinary Medicine and 26 samples from Kansas State University. B-cell lymphomas contributed to 64% of the population at Tufts and 35% from KSU. T-cell lymphomas were more common in the population at KSU (35%) compared to Tufts (21%). There were five mixed cell phenotypes from Tufts (15%) and eight from KSU (30%). Complete clinical response to chemotherapy was highest for the B-cell lymphoma group (63%) followed by the mixed phenotype group (62%) and the T-cell group (31%). Likewise, the T-cell group had the highest percentage of dogs that did not respond to chemotherapy (7 out of 16). A total of nineteen cases had concurrent leukemia (Table 2)

Table 2
Immunophenotype of lymphoma samples

	Total	Clinical response				Cases with leukemia
		CR	PR	NR	NT	
B-cell lymphoma cases	30	19	3	2	2	8
CD79a+IgM+CD21+	15	8	2	1	1	5
CD79a+IgM+CD21–	1	1				1
CD79a+IgM–CD21+	5	4				1
CD79a ^a	1		1			
IgM+CD21 ^a	3	2		1		1
CD21+IgM–	5	4		1		
T-cell lymphoma cases	16	5	2	7	–	5
CD3 ^a	2	1		1		1
CD3+CD4+CD8–	13	4	2	5		3
CD3+CD4+CD8+	1			1		1
Mixed B- and T-cell cases	13	8	2	1	2	6
CD3+CD79a+CD21–	8	6	1	1		5
CD3+CD79a–CD21+	3	1	1		1	1
CD3+CD79+CD21+	1	1			1	

^a Reduced cellularity limited antibody combinations; CR: complete response to therapy; PR: partial response to therapy; NR: no response to therapy; NT: no treatment.

including eight B-cell cases, five T-cell cases, and six mixed cell cases.

3.3. B-cell lymphomas

Of the B-cell cases, expression of CD79a or CD21 ranged from 65 to 100% of the lymphocyte population with <30% of the cells expressing CD3. The immunophenotyping results of ten cases from Tufts were consistent with the CD79a+CD3– staining pattern of lymph node sections determined by immunohistochemistry. In 21 cases in which CD79a, CD21, and IgM could be evaluated, 15 cases showed expression of all three markers supporting a mature B-cell phenotype. Five of these also had leukemia. Dual-immunofluorescent labeling for CD21 and CD79a was performed on six cases and demonstrated co-expression of CD21 and CD79a on 43–81% of the B-cells. Fig. 2 illustrates the staining pattern of a B-cell lymphoma case in which predominately large cells were CD21+IgM+CD79a+, but CD3–. Dual immunofluorescence staining confirmed that the CD79a+ cells were negative for CD3 (Fig. 2, lower row, middle plot) and identified a subset (72%) of CD79a+CD21+ cells, indicating a predominance of a differentiated B-lymphocyte population (Fig. 2, lower row, right-hand plot). One case had an immature B-cell phenotype, CD79a+IgM+CD21–,

whereas five cases had a mature phenotype CD79a+CD21+ but lacked IgM expression. Staining for CD79a could not be performed in eight cases that had limited cellularity. Of these cases, three were CD21+IgM+, whereas five were CD21+IgM– (Table 2). Surface IgG was detected in three out of the five that were CD21+IgM– (not shown) indicating Ig class switching. Three B-cell lymphoma cases expressed the stem cell marker (CD34) ranging from 37% to 55% of the cells. Fig. 3a shows the staining pattern of a B-cell case expressing CD34 on large cells (>450 FSC-H channels) which also expressed CD21, IgM, and dimly expressed CD79a. This preparation also had populations of medium size lymphocytes (300–450 FSC-H channels) that were CD3+ and few small lymphocytes that expressed CD21, IgM, and CD34. We hypothesized that the medium size cells were resident T-lymphocytes that contributed to the polyclonal TCR γ banding pattern noted in lane 4 of the PCR reaction (Fig. 3b, lane 2). This CD34+ case was confirmed to be of B-cell lineage by detection of an immunoglobulin gene rearrangement (Fig. 3b, lane 2). A second CD34+B-cell lymphoma case also had a clonal B-cell antigen gene rearrangement (not shown). A total of four B-cell cases were confirmed by PCR methods to be of B-cell lineage and four other cases were CD79a+CD3– by immunohistochemistry (data not shown).

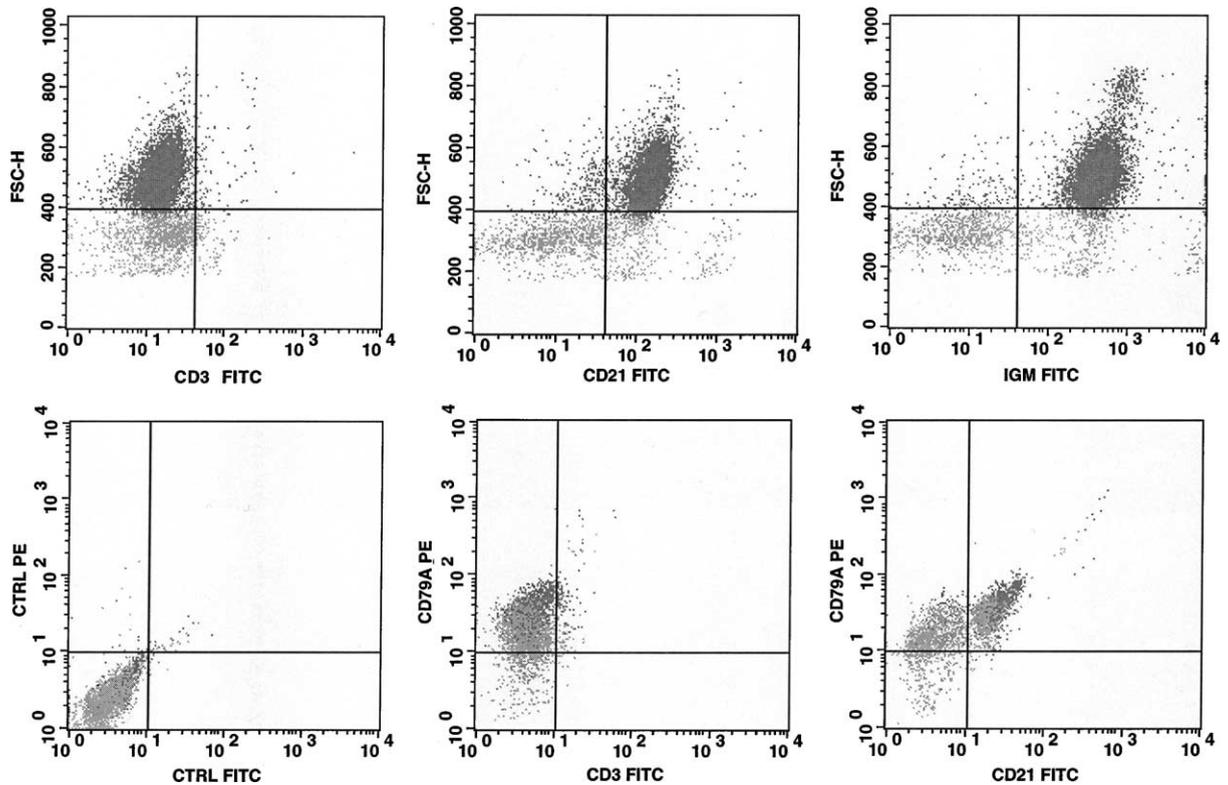


Fig. 2. Flow cytometry plots (FSC-H vs. FITC, top row) and dual immunofluorescent plots (PE vs. FITC plots, bottom row) from a dog with a B-cell lymphoma that had CD3-CD21+IgM+CD79a+ lymphocytes. Horizontal line separates small from large lymphocytes based on FSC-H channels (y-axis, linear scale). Vertical line is placed to the right of cells labeled with negative control antibody (CTRL FITC, not shown). For dual immunofluorescent plots, quadrants (horizontal and vertical lines) were placed on cells labeled with negative control antibodies (CTRL PE or FITC, lower row, left-hand plot). Based on dual immunofluorescent plots neoplastic lymphocytes were CD79a+CD3– (upper left quadrant, second row, middle plot) and CD79a+CD21+ subset (upper right quadrant, right-hand plot).

3.4. T-cell lymphomas

Sixteen cases were T-cell defined by T-cell marker expression (CD3, CD4, or CD8) ranging from 75% to 97% of the population with <20% of the cells expressing B-cell markers. Thirteen cases were CD3+CD4+CD8–. One case with multicentric lymphoma including ocular involvement was CD3+CD4+CD8+. This phenotype profile was indicative of an early thymocyte differentiation stage. Five cases were tested and confirmed by PCR to have a clonal T-cell antigen gene rearrangement. Fig. 4 illustrates a typical staining pattern for a T-cell lymphoma in which medium to large lymphocytes were CD3+CD4+CD79a– (350–750 FSC-H channels). There were few small cells in this preparation that brightly stained for CD8, surface IgM or CD79a

(250– 350 FSC-H channels) supporting residual populations of B- and T-lymphocytes.

3.5. Mixed cell cases

The remaining 13 cases expressed both B- and T-cell markers and were designated as mixed phenotype. Many of the cases expressed combinations of CD79a or CD21 and CD3 or other T-cell markers at $\geq 60\%$ for each marker, with totals exceeding 100%. One of these cases demonstrated over 80% of the lymphocytes were large (400–1000 FSC-H channels) and CD45+CD3+CD4+CD21+CD14+CD79a+ (Fig. 5a). The expression of CD45, CD4, CD21, and CD14 had less fluorescent intensity than CD3 and CD79a. This case was determined to be a T-cell lymphoma based on PCR results that identified an oligoclonal pattern for a TCR γ

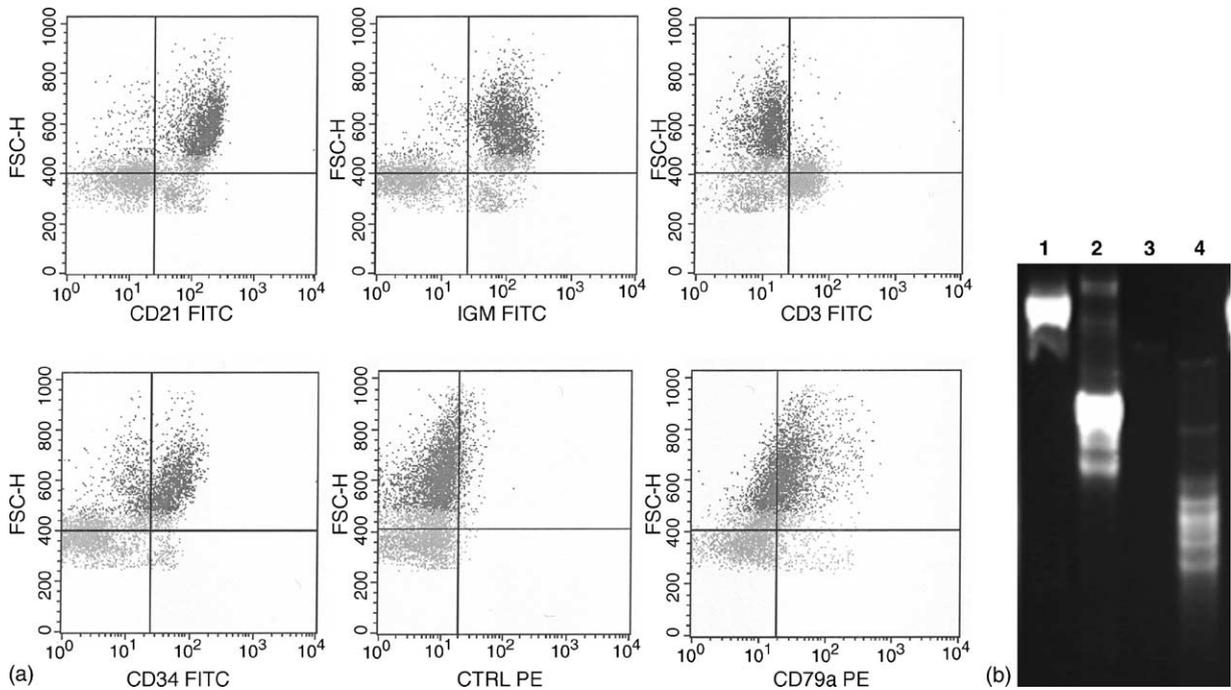


Fig. 3. Flow cytometry plots (FSC-H vs. FITC and FSC vs. PE) (a) and PCR results (b) from a dog with a B-cell lymphoma. (a) Scatter plots indicated the presence of large lymphocytes that brightly expressed CD21, IgM, and CD34 with dim expression of CD79a. There were subsets of medium lymphocytes that expressed CD3 and small lymphocytes that expressed CD21 and IgM. Horizontal line separates small from large lymphocytes based on FSC-H channels (y-axis, linear scale). For top row, vertical line is placed to the right of cells labeled with negative control antibody (CTRL FITC, not shown). For CD79a, vertical line is placed to the right of cells labeled with negative control antibody (CTRL PE, bottom row, middle plot). (b) PCR results demonstrate amplification of single DNA product from a clonal population of cells that had a gene rearrangement for Ig major (lane 2). Lane 1 is the positive control for DNA. Lane 3 is negative using Ig minor primers. Lane 4 shows amplification of DNA from a polyclonal population of T-cells expressing the TCR γ gene.

gene rearrangement (Fig. 5b, lane 4). Eight of the thirteen cases expressed CD3 and CD79a with a bright fluorescent intensity. Three cases expressed CD3 and CD21 (Table 2), whereas one case expressed all three CD3, CD79a and CD21 markers. Nine of the CD3+CD79a+ cases also expressed CD4 in $\geq 60\%$ of the lymphocytes.

Since expression of these CD molecules suggested populations of neoplastic cells expressing T- and B-cell markers simultaneously, dual immunolabeling was performed to confirm co-expression of either CD79a and CD3 or CD21 and CD3 in a total of six cases. Dual immunofluorescence analysis identified co-expression of CD79a and CD3 in four out of five cases, and CD21 and CD3 co-expression in one case. One case had CD79a+ and CD3+ cells; however, the two populations stained mutually exclusive for these markers. Seven cases that had a mixed cell phenotype

were analyzed for clonality using PCR. Four cases had TCR γ rearrangement, two had Ig rearrangement, and one was PCR negative for T- and B-receptor rearrangements. Two dogs confirmed to have a clonal population of T-cells in the lymph node aspirate also had T-cells (20–30%) infiltrating the bone marrow as determined by immunophenotyping (data not shown).

Fig. 6 illustrates a case in which 94% of the cells expressed CD3, 90% expressed CD4, but CD21 expression accounted for only 9% of the population. Additionally, expression of IgM on these cells was very low (11%, data not shown). Dual immunofluorescence analysis showed 73% of the lymphocytes co-expressed CD79a and CD3 (Fig. 6, bottom row, right-hand plot). Using color gating to distinguish small (green) from large (pink) lymphocytes on a FSC-H versus SSC-H plot, CD79a and CD3 co-expression was detected principally on large lymphocytes, but

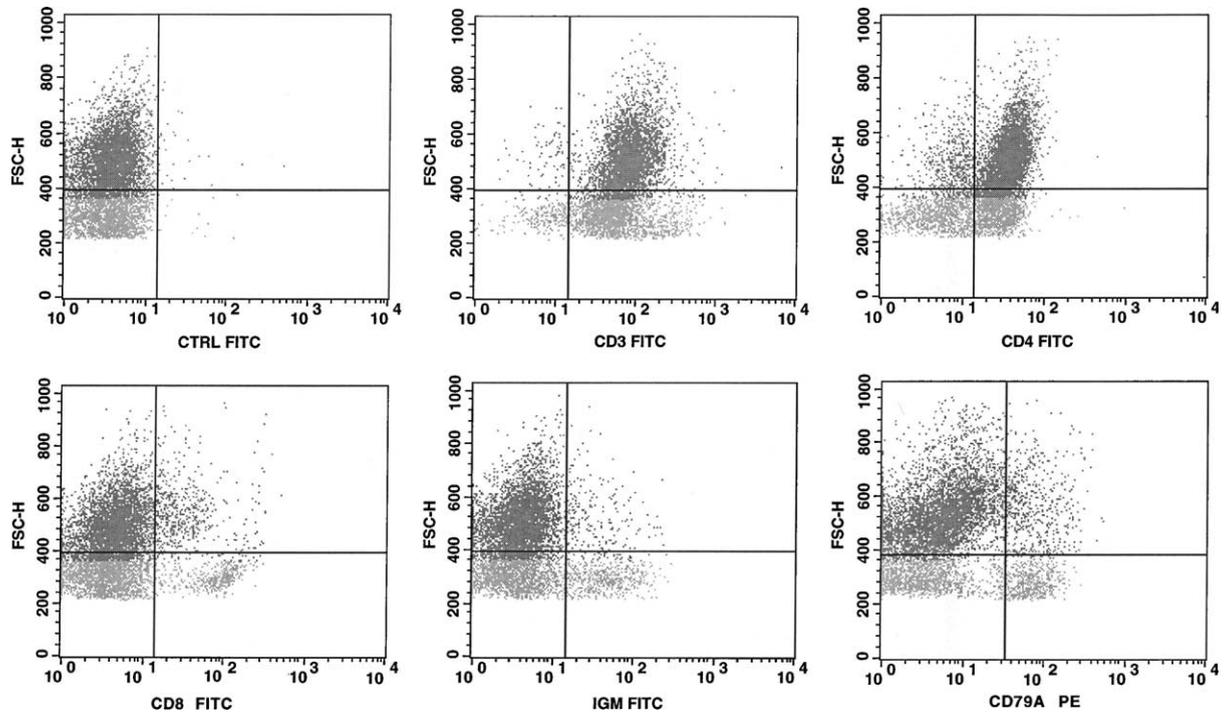


Fig. 4. Flow cytometry plots (FSC-H vs. FITC and FSC-H vs. PE) of a lymph node sample from a dog with a T-cell lymphoma had small and large lymphocytes expressing CD3 (top row, middle plot) and CD4 (top row, right-hand plot). A subset of small lymphocytes brightly expressed CD8 (bottom row, left-hand plot) and a subset of small lymphocytes expressed IgM (bottom row, middle plot) and CD79a (bottom row, right-hand plot). Horizontal line separates small from large lymphocytes based on FSC-H channels (linear scale, y-axis). Vertical line is placed to the right of cells labeled with negative control antibody (CTRL FITC, top row, left-hand plot) or PE-conjugated antibody (not shown).

few small lymphocytes also co-expressed these molecules indicating that a majority of the total cells in the sample were likely neoplastic.

Four cases expressed CD3 and CD21 on >60% of the lymphocytes. Two of these cases also expressed CD4, CD8 and CD14. One case expressed IgM. Fig. 7a illustrates the flow cytometric staining pattern of one case that was CD3+CD8+CD14+CD21+IgM+CD79a+ principally on large cells (400–700 FSC-H channels, note the cells decreased in size following staining for CD79a). In contrast to the T-cell cases that aberrantly expressed B-cell molecules, this case was determined to have a B-cell receptor gene (Ig heavy chain) rearrangement by PCR (Fig. 7b, lane 2). One CD3+CD21+ case was negative for both T- and B-cell receptor rearrangements by PCR and expressed CD34, the stem cell marker (data not shown). Fig. 8 illustrates the usefulness of the dual color immunofluorescence technique to identify lymphocyte popu-

lations that express CD21 exclusive of CD3 in a case of a B-cell lymphoma (left-hand column) or CD3 exclusive of CD21 in the case of a T-cell lymphoma (middle column). A mixed cell lymphoma case had co-expression of both CD3 and CD21 surface markers (right-hand column), which was determined to be of B-cell lineage based on detection of a B-cell receptor rearrangement (data not shown). The size of the neoplastic cells in this mixed cell case was large even though size is not apparent by the dual immunofluorescent staining plot.

4. Discussion

In this study, we determined the CD molecule expression profile of 59 cases of canine lymphoma/leukemia. This disease is a malignant disorder derived from the clonal proliferation of lymphoid precursor

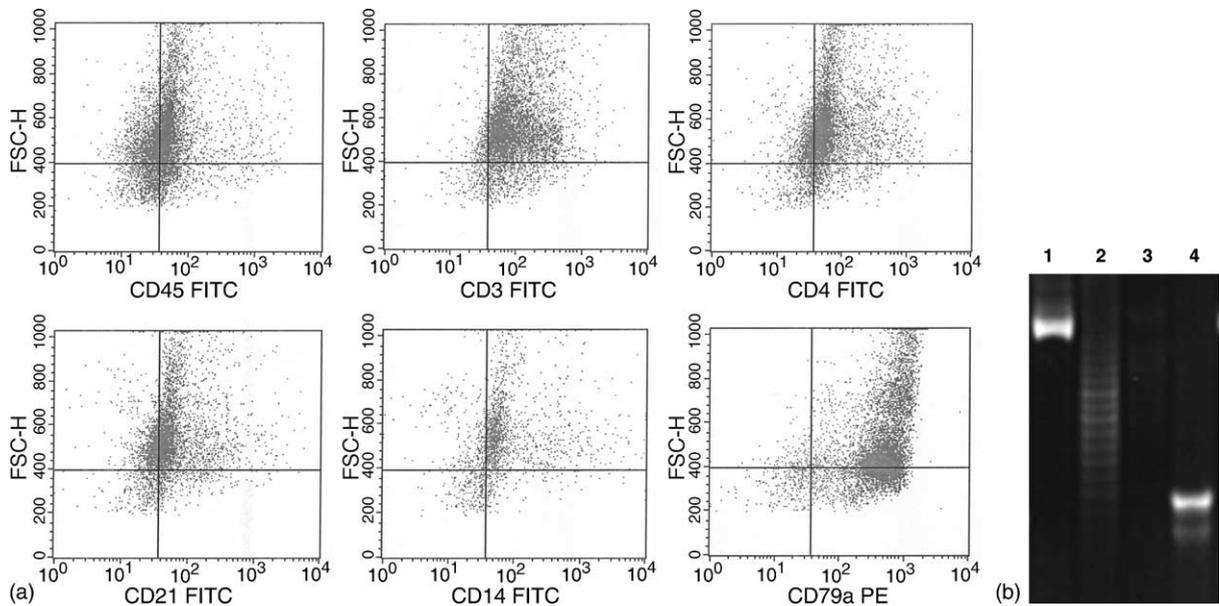


Fig. 5. Flow cytometry plots (FSC-H vs. FITC, FSC-H vs. PE) (a) and PCR results (b) from a dog with lymphoma. (a) Predominantly large lymphocytes are present with dim expression for CD45, CD3, CD4, CD21, and CD14, but bright expression for CD79a. Horizontal line separates small from large lymphocytes based on FSC-H channels (linear scale, y-axis). Vertical line is placed to the right of cells labeled with negative control antibody (CTRL FITC or PE, not shown). (b) PCR results demonstrate amplification of two DNA products from a population of cells that had a bi-clonal gene rearrangement for TCR γ gene (lane 4). Lane 1 is the positive control for DNA. Lanes 2 and 3 had amplification of DNA from a polyclonal population of B-cells using primers for the Ig major and minor gene.

cells. Clinical studies have shown that dogs with T-cell lymphoma typically have a poorer prognosis and are less responsive to treatment. Our study supports this observation. Using multiple combinations of CD antigen expression and forward light scatter properties of the cells, we classified lymph node cells obtained from dogs, both healthy and affected with lymphoma, into B-cell, T-cell, and mixed phenotypes. Using values obtained from healthy dog samples, a cutoff value of $\geq 60\%$ of the cells labeling for either a B- or T-cell marker was used to interpret the staining patterns of tumor preparations. This guideline is similar to the criteria used for cytologic assessment of needle aspirates from dogs in which a diagnosis of malignant lymphoma can be confidently made when $>50\%$ of the cells have immature features (Cowell et al., 1999). It should be noted that expressing flow cytometry data for lymph node populations as percent positive alone can be misleading and contribute to erroneous interpretations. At many human medical institutions a 20% cutoff is often employed, however, it is unclear how this value was established. It is recommended that

the “percent positive” per antibody be viewed in the context of the forward light scatter properties of the stained and nonstained cells and the fluorescent intensity of the stained populations (Nguyen et al., 2003). Applying this approach allows one to distinguish normal cells from neoplastic cells, so that over-interpretation of the immunophenotyping results is less likely.

We have shown with multiparameter flow cytometric analysis how the forward light scatter and multifluorochrome staining patterns provide the CD expression profile of large and small lymphocytes in both healthy and neoplastic samples. This information is useful in identifying the presence of reserve cells when a neoplastic population predominates in the sample. Furthermore, dual immunofluorescent labeling confirms the presence of cells that co-express CD molecules and distinguishes aberrant cells from resident cells that have defined lineage profiles. Flow cytometric features most suspicious of malignancy include not only aberrant antigen expression but also the loss of or markedly dimmed expression of CD45 in

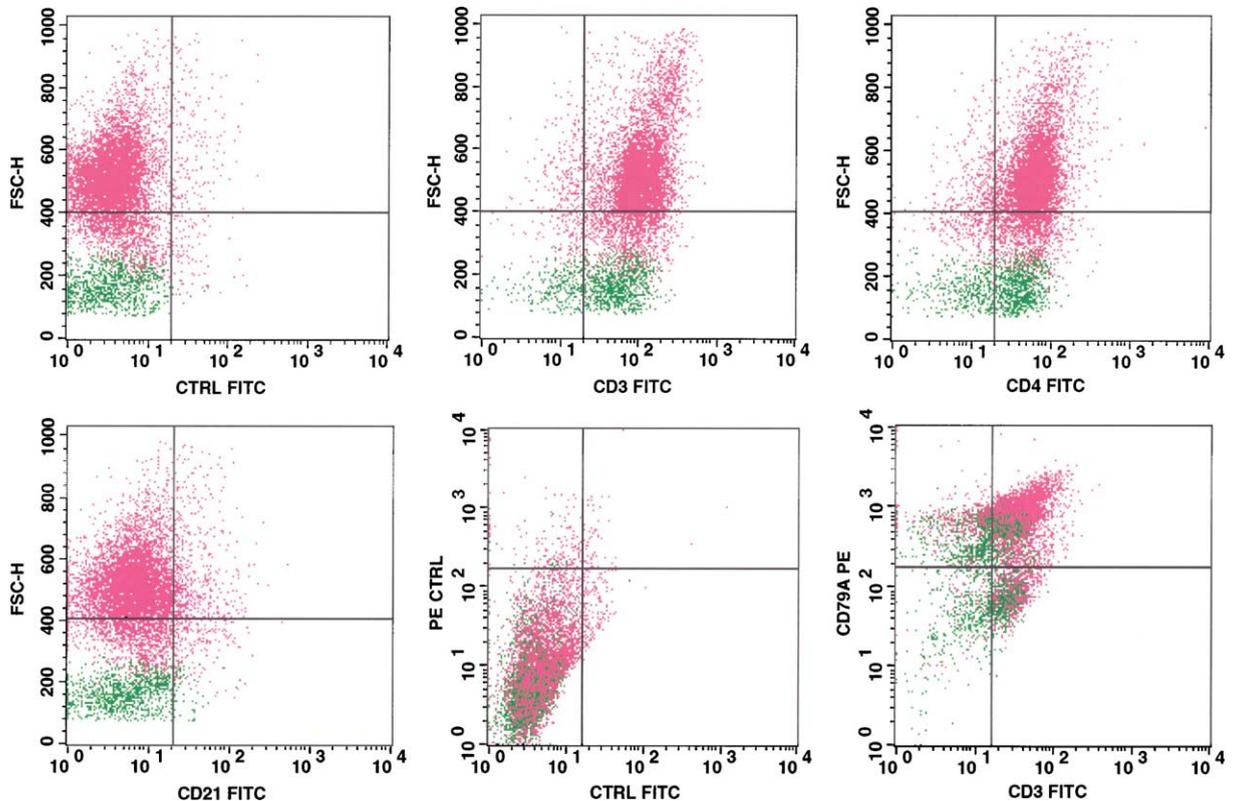


Fig. 6. Flow cytometry plots (FSC-H vs. FITC) and a dual immunofluorescence plot (PE vs. FITC) from a dog with lymphoma. Large and small lymphocytes are CD3+CD4+CD21-. Horizontal line separates small from large lymphocytes based on FSC-H channels (linear scale, y-axis). Vertical line is placed to the right of cells labeled with negative control antibody (CTRL FITC, top row, left-hand plot). A predominant population of large cells (pink) co-express CD79a and CD3 based on dual immunofluorescent labeling (second row, right-hand plot, upper right quadrant), with few small (green) and large cells (pink) expressing only CD3 (lower right quadrant). Quadrants were placed on cells labeled with negative control antibodies (PE CTRL and CTRL FITC, bottom row, middle plot). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

conjunction with changes in cell size (Gorczyca et al., 2002; Nguyen et al., 2003; Avery and Avery, 2004). Dim expression of CD45 together with aberrant CD molecule expression was detected in one case in this report. In support of this finding, a report from Colorado state University described 11 out of 26 canine T-cell leukemias had lost either CD45 or pan-T cell antigens (CD5, CD4, or CD8) (Avery and Avery, 2004). A hallmark of human T-cell leukemias is their tendency to lose expression of normal T-cell antigens, or display aberrant combinations (Jennings and Foon, 1997).

The majority of B-cell lymphomas in this report expressed CD79a, CD21, and IgM or combinations thereof. Most of these cases appeared to represent a

mature B-cell phenotype based on expression of CD21. One case that did not express CD21 but expressed CD79a and IgM likely represented arrest at an immature stage of B-cell maturation. CD79a expression precedes immunoglobulin heavy-chain gene rearrangement during B-cell development and is expressed in human lymphoma/leukemias arrested at the pre-B-cell stage (Chu and Arber, 2001). In our study, four out of five canine lymphoma cases that were CD79a+CD21+IgM- expressed surface IgG suggesting a mature state in which class switching had occurred. Since antibodies to CD38, a specific antigen expressed by plasma cells, is not available for dog lymphocytes, CD79a is a useful marker to distinguish immature B-cells from plasma cells tumors. In

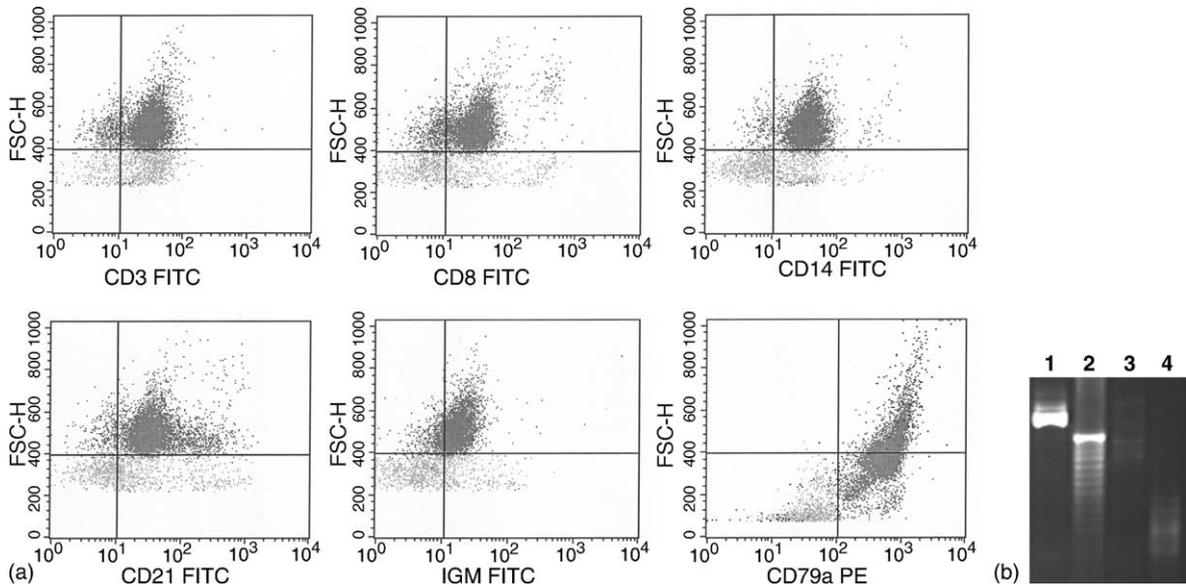


Fig. 7. Flow cytometry plots (FSC-H vs. FITC and FSC-H vs. PE) (a) and PCR results (b) from a dog with lymphoma. (a) Large lymphocytes were CD3+CD8+CD14+CD21+IgM+CD79a+. Horizontal line separates small from large lymphocytes based on FSC-H channels (linear scale, y-axis). Vertical line is placed to the right of cells labeled with negative control antibody (either CTRL FITC or PE, not shown). (b) PCR results demonstrate amplification of a single DNA product from a population of cells that had a clonal gene rearrangement for Ig major gene (lane 2). Lane 1 is the positive control for DNA. Lanes 2–4 show faint amplification of DNA from polyclonal populations of residual T- and B-cells using primers for the Ig minor and TCR γ genes.

humans, surface Ig and CD21 expression is lost in myeloma/plasmacytomas while CD79a expression persists (Chu and Arber, 2001). Further defining the differentiation stage of canine B-cell neoplasms will occur as more antibody reagents to other B-cell antigens become available.

An interesting finding was three lymphoma cases in which CD34, a glycosylated surface glycoprotein expressed on stem cells, was detected on a mature B-cell phenotype. In one study of human lymphoma cases, 15% of the B-cell lymphomas that expressed CD19 or CD20 also expressed CD34. These cases were described as high grade lymphomas (Schmidt et al., 1999). Because CD19 and CD20 are expressed at earlier stages of B-cell development than CD21, expression of CD34 would be expected. However, expression of CD34 on a CD21+ lymphoma (mature B-cell phenotype) is unusual and suggests aberrant expression of CD34. A recent study reported the lack of CD34 expression in 299 canine lymphomas (including B- and T-cell lymphomas) and common CD34 expression in 39 acute leukemias (Vernau, 2004). The association of the CD34 phenotype with

clinical grade or progression of disease in dogs with lymphoma is not known at this time.

The majority of T-cell lymphomas in our study and in other reports were of the CD4 subset (Culmsee et al., 2001; Ruslander et al., 1997). This is in contrast to dogs with chronic lymphocytic leukemia that have a predominant CD8 phenotype (Ruslander et al., 1997; Vernau and Moore, 1999). As reported in a low percentage of dogs with lymphoproliferative disease (Ruslander et al., 1997), we detected a case that expressed all three T-cell antigens CD3, CD4, and CD8. In agreement with prior reports, we show that dogs with T-cell lymphoma had the shortest survival times.

In our study, we identified thirteen cases with a mixed immunophenotype and further immunostaining was used to identify co-expression. Using dual immunolabeling techniques several cases had populations of cells that simultaneously expressed CD3 and CD79a or CD3 and CD21, whereas one case was shown to have populations exclusively CD3+ and CD79a+. Although dual immunofluorescent staining can identify cases that are rich in both T- and B-cell

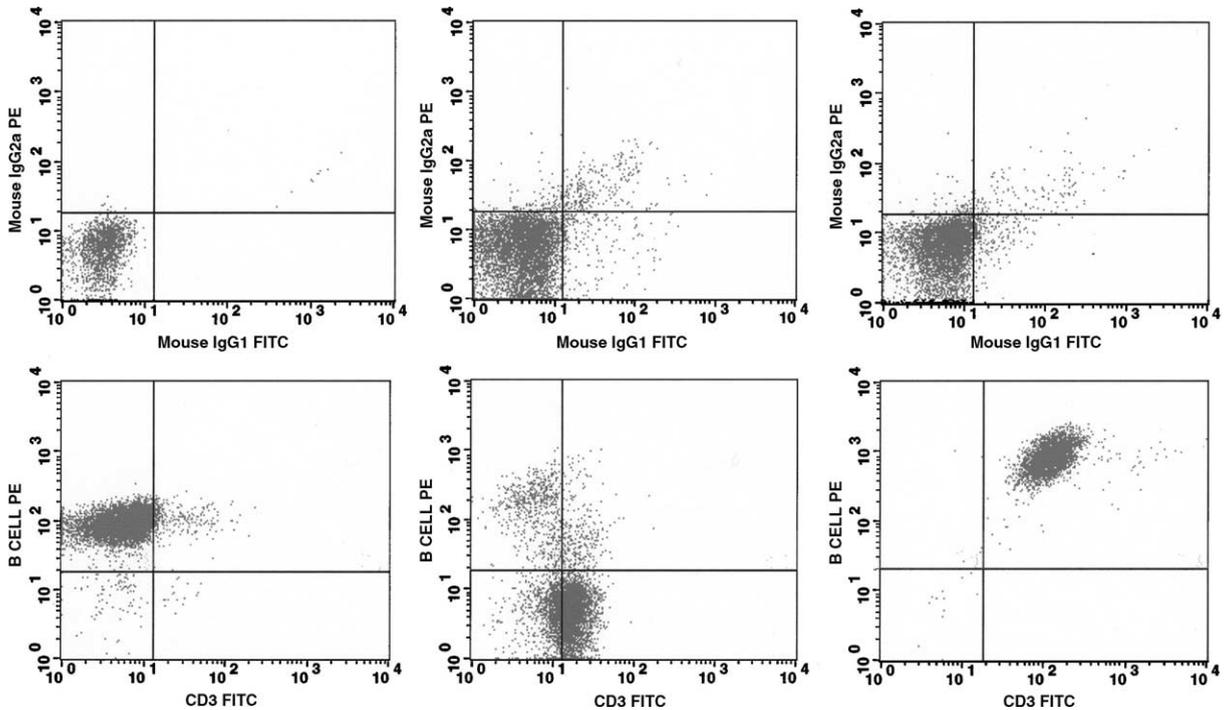


Fig. 8. Dual immunofluorescent staining with isotype controls (top row) and B-cell (CD21) PE and CD3 FITC antibodies (bottom row) of three types of lymphoma. A B-cell lymphoma expresses CD21 exclusive of CD3, upper right quadrant (left-hand column). A T-cell lymphoma predominantly expresses CD3 (lower right quadrant), but few cells express CD21 (upper left quadrant) (middle column). A mixed cell lymphoma stains brightly for both CD21 and CD3 simultaneously, upper left quadrant (right-hand column). Horizontal line separates the nonstaining cells using isotype control antibody (mouse IgG2a PE) from the CD21 PE stained cells. Vertical line separates the nonstaining cells using isotype control antibody (mouse IgG1 FITC) from the cells stained with CD3 FITC.

subsets, diagnostic interpretation of the immunophenotyping results becomes difficult when one or more of the T- or B-cell antigens are detected on the neoplastic cells.

Using flow cytometry and immunofluorescent labeling with a single fluorochrome two separate studies of canine lymphoma identified 44% (27/62) and 55% (22/40) of the case samples labeled with pan-T and surface immunoglobulin markers (Appelbaum et al., 1984; Greenlee et al., 1990). Later studies surveyed a total of 134 canine cases of lymphoma and showed exclusive expression of B- or T-cell markers on lymphoma cells using immunohistochemistry (Fournel-Fleury et al., 1997). Culmsee et al. (2001) immunophenotyped lymph node aspirate samples from 30 dogs using flow cytometry and dual immunofluorescent labeling for CD79a and CD3. Although none of the cases in their report showed co-expression for CD79a and CD3, their

methods differed from our study in that they used a FITC conjugated human antibody specific to the cytoplasmic portion of CD3 together with the PE conjugated CD79a antibody (Culmsee et al., 2001). Intracellular staining for two cytoplasmic molecules using two fluorochrome conjugated antibodies can be difficult, since PE (a phycobiliprotein of high molecular weight) typically precludes the entry of a second antibody (Holmes et al., 2002). However, using microwave techniques to retrieve tissue antigens and immunohistochemistry, co-expression of cytoplasmic CD79a and CD3 was identified in a lymph node section from a dog with aggressive T-cell lymphoma/leukemia and chromosomal alterations (Thomas et al., 2001).

In human cases CD79a/CD3 co-expression has been reported in 40% of acute T-lymphoblastic leukemia/lymphoma using double staining immunofluorescence and western blotting (Lai et al., 2000;

Pilozzi et al., 1998). T- and B-cell co-expression in humans has been associated with reduced survival time and angioblastic T-cell lymphomas (Attygalle et al., 2004; Yao et al., 2001; Lai et al., 2000; Pilozzi et al., 1999). Several cases were assigned to the T-cell category because they expressed early or common thymocyte markers (CD7 and CD1a), but not B-cell markers such as CD19 or CD22 (Pilozzi et al., 1998), whereas others were determined to have T-cell antigen gene rearrangements by PCR (Pilozzi et al., 1999; Lai et al., 2000; Yao et al., 2001). The single canine case reported with CD79a and CD3 antigen co-expression by immunohistochemistry had alterations in several chromosomes (Thomas et al., 2001).

In our study expression of CD21 was observed in three lymphoma cases that also expressed T-cell markers (i.e. one was CD3+CD4+, and two were CD3+CD4+CD8+). CD21 is a complement receptor (C3d) principally expressed on mature B-lymphocytes. However, CD21 is thought to be a target for the Epstein Barr virus and is expressed in immature thymocytes at a high density on CD4–CD8– double negative thymocytes and during early acquisition of CD4 (Fischer et al., 1999). CD21 expression on these cases reflects aberrant expression on a mature T-cell phenotype because the lineage markers CD4+ and CD8+ are associated with maturation. Including additional T-cell markers that are expressed on earlier stages of thymocytes in a multi-color format (i.e. terminal deoxynucleotidyl transferase, CD7, and CD1a) would help identify CD expression profiles that fit with early and common thymocyte stages of development that typify lymphoblastic lymphoma in humans (Rezuke et al., 1997). Expression of many of the mature CD molecules appeared to be aberrant in these three cases that co-expressed CD3 and CD21, for PCR testing revealed that one had a TCR γ clonal rearrangement, and two had an Ig clonal rearrangement, therefore the antigen expression profiles were not predictive of the lineage.

Based on the reports in the literature, one could hypothesize that the canine lymphoma cases with T- and B-cell marker co-expression represent aggressive T-cell lymphoma/leukemias. Further studies are essential to fully evaluate whether aberrant expression of CD molecules correlates with progression of the disease or survival rate in dogs. However, using PCR we identified T-cell receptor gene rearrange-

ment in three co-expressing cases and a B-cell receptor rearrangement in two cases. Uniquely, one B-cell case also expressed CD8 together with CD3 and CD14. Aberrant expression of CD8 antigen has been reported in 64% of humans with B-cell chronic lymphocytic leukemia (Espinosa et al., 2003). A strict marker for macrophages, CD14, can be expressed by human pro-B-cells under the appropriate environmental conditions in vitro (Reynaud et al., 2003), and reported to be expressed in unclassified acute leukemias, immature and mature B-cell leukemias, multiple myeloma, and nonHodgkin's lymphoma (Nakase et al., 1996). Precursor B-cell leukemias in humans that aberrantly express CD antigens are associated with chromosomal translocations and poor prognosis (Jennings and Foon, 1997).

Molecular methods to identify clonal B- and T-cell receptor gene are essential in determining lineage and clonality. However, the PCR assay has a 9–15% false negative rate due to the lack of primer specificity for the particular gene rearrangement (Burnett et al., 2002). Aberrant expression of CD molecules in human lymphoma/leukemias has been proposed to be a consequence of lineage infidelity. In fact, T- and B-cell receptor gene rearrangements have been identified in acute myeloid leukemias indicating the pluripotential nature of hemic derived cells (Schmidt and Przybylski, 2001; Schmidt et al., 1999). Aberrant CD antigen expression can be used as strong support for a diagnosis of leukemia or lymphomas because reactive lymphocytes typically retain their antigens (Avery et al., 2004; Rezuke et al., 1997). Although the proportion of dogs in our study that also had leukemic states was greatest for the mixed cell lineage, these dogs had a higher rate of CR than dogs in the T-cell lymphoma group. The prognostic implications of alterations in CD antigen expression in dogs with lymphoma/leukemia are not known and need further study.

5. Conclusions

Collectively, these results illustrate how multiple assays to determine cell type and clonality complement each other and when performed together improve the accuracy of the interpretation of the flow

cytometry immunophenotype profile. In this report, we illustrate with several cases differential CD antigen expression among neoplastic and reserve cells in the same sample. Moreover, we report here for the first time aberrant expression of CD molecules in canine lymphomas using multiparameter flow cytometry. These aberrant cases suggest that lineage infidelity occurs in canine lymphoma/leukemia, as described in human cases with lymphoproliferative disorders. These observations support the finding that no marker has absolute lineage specificity and that immunophenotypic studies should be performed with panels of monoclonal antibodies and molecular methods to identify lineage, clonality, and gene rearrangements. Moreover, detection of aberrant CD antigen expression in lymph node samples is a good indicator for the presence of neoplastic cells.

Acknowledgements

The authors wish to thank Heather Heeb, Stacy Santoro, and Mindy Graves for their contributions in the case submission.

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