
Use of the Cell-Dyn 3500 to Predict Leukemic Cell Lineage in Peripheral Blood of Dogs and Cats

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Background — Morphology and cytochemistry are the foundation for classification of leukemias in dogs and cats. Advances in automated hematology instrumentation, immunophenotyping, cytogenetics, and molecular biology are significantly improving our ability to recognize and classify spontaneous myeloproliferative and lymphoproliferative disorders. **Objective** — The purpose of this study was to assess the utility of flow cytometry–based light scatter patterns provided by the Cell-Dyn 3500 (CD3500) automated hematology analyzer to predict the lineage of leukemic cells in peripheral blood of dogs and cats. **Methods** — Leukemic cells from 15 dogs and 6 cats were provisionally classified using an algorithm based on the CD3500 CBC output data and were subsequently phenotyped by enzyme cytochemistry, immunocytochemistry, indirect flow cytometry, and analysis of antigen receptor gene rearrangement. **Results** — The algorithm led to correct predictions regarding the ontogeny of the leukemic cells (erythroid/megakaryocytic potential, myeloid leukemia, monocytic leukemia, chronic granulocytic leukemia, lymphoid leukemia) in 19/21 animals. Mismatches in the WBC impedance count and the WBC optical count in conjunction with microscopic assessment of blasts in the blood were useful for predicting myeloproliferative disorders with erythroid or megakaryocytic potential. The leukocyte light scatter patterns enabled distinction among myeloid leukemias (represented by acute myelomonocytic leukemia, acute monocytic leukemia, chronic granulocytic leukemia) and lymphocytic leukemias (including acute and chronic lymphocytic leukemias). One case of acute lymphocytic leukemia was misidentified as chronic lymphocytic leukemia. **Conclusions** — Algorithmic analyses can be applied to data generated by the CD3500 to predict the ontogeny of leukemic cells in the peripheral blood of dogs and cats. This rapid and quantitative technique may be used to improve diagnostic decisions, expand therapeutic choices, and increase prognostic accuracy. (*Vet Clin Pathol.* 2002;31:167-182)

Key Words: Cell-Dyn 3500, automated hematology analyzer, flow cytometry, leukemia, light scatter patterns, lymphoma, phenotyping

Light microscopic morphology and enzyme cytochemistry are the foundation for the French-American-British (FAB) classification of hematopoietic malignancies.¹ Morphology-based classification of leukemias requires meticulous examination of bone marrow or peripheral blood by an experienced hematopathologist, and even when blasts are identified, their lineage may not be easily distinguishable. Phenotyping by enzyme cytochemistry and immunocytochemistry also can be used to assist in the determination of leukemic cell lineages, but these techniques are specialized and can be time consuming and expensive.²⁻⁷ Conversely, flow cytometric analysis is easy to perform, rapid, and economical, and it can be used to quantify normal and abnormal cells in the blood based on the unique light scatter properties of leukocytes.⁸⁻²⁰

Dogs and cats are commonly afflicted with spontaneous leukemias that include a full spectrum of myeloproliferative and lymphoproliferative disorders with

many similarities to their human counterparts, both in clinical presentation and hematopathologic behavior.²¹⁻²³ The ability to use laboratory instrumentation to identify these cells was recently enhanced by the introduction of automated analyzers with specific adaptations for veterinary species. One of the first among these was the Abbott Cell-Dyn 3500 (CD3500; Abbott Laboratories, Abbott Park, Ill, USA), which uses principles of electrical impedance, laser light scatter, and multiparameter flow cytometry.²⁴ Three independent measurements are used by the CD3500 to obtain the hematologic parameters reported with a CBC: an impedance-based RBC count and platelet count, a WBC impedance count (WIC), and a WBC optical count (WOC). A veterinary software package enables the CD3500 to analyze samples from common veterinary species using calibration settings developed by the manufacturer.^{12,25}

For this study, we constructed an algorithm based on output data from the CD3500 CBC to predict the

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ontogeny of leukemic cells in canine or feline peripheral blood samples. The clinical utility of this algorithm was tested in a population of 15 dogs and 6 cats by comparing the predictions derived from the CD3500 data with the final diagnoses based on enzyme cytochemistry and immunophenotyping of the malignant cells.

Materials and Methods

Patient selection

Samples for this study were obtained from 15 consecutive client-owned dogs (dogs 1-15) and 6 consecutive client-owned cats (cats 1-6) seen at the Texas Veterinary Medical Center and/or referred to the Clinical Pathology Laboratory of Texas A&M University between May 1996 and December 1998. These animals were diagnosed with leukemia or lymphoproliferative disease

with circulating malignant lymphocytes. The following results obtained from CD3500 analysis of blood triggered further evaluation of the flagging diagnostics, including numbers of blasts, immature granulocytes (IG), and bands, and microscopic examination of Wright's-stained blood smears by technical staff and one of the participating pathologists:

1. Flagging diagnostics indicated $\geq 2\%$ blasts.
2. WBC count exceeded the reference range used at the Clinical Pathology Laboratory (dogs: 6000-17,000 WBCs/ μ L; cats: 5500-19,500 WBCs/ μ L) and
 - a. granulocytosis (\pm monocytosis) of 17,000-60,000 WBCs/ μ L (dog) or 19,500-60,000 WBCs/ μ L (cat) with immature granulocytes and blast alert flags or
 - b. granulocytosis (\pm monocytosis) of $>60,000$ WBCs/ μ L with neutrophilia and a significant left shift (flag-

Table 1. Patient characteristics and inclusion criteria.

Animal	Breed	Age	Sex*	Alert Flags†	Criteria for Inclusion‡
Dog 1	Greyhound	6 y	F	NRBC, DFLT (NE)	WIC:WOC = 2.12; 13% blasts (manual differential)
Dog 2	Dalmatian	4 y	S	NWBC, DFLT (NLMEB)	27,200 WBC/ μ L; 17% bands, 36% IG
Dog 3	Golden Retriever	4 y	S	DFLT (NLME)	105,000 WBC/ μ L; 23% bands, 18% IG
Dog 4	Golden Retriever	3 y	S	NWBC, DFLT (NLMEB)	42,400 WBC/ μ L; 77% blasts
Dog 5	Irish Setter	3 y	M		36,100 WBC/ μ L; 71% blasts
Dog 6	Golden Retriever	4 y	S	FWBC, DFLT (LM)	20,200 WBC/ μ L; 63% mononuclear cells (unimodal), 20% blasts
Dog 7	Mixed Breed	11 y	M	DFLT (NLME)	93,100 WBC/ μ L; 95% mononuclear cells (unimodal), 49% blasts
Dog 8	Shih Tzu	13 y	C	FWBC	48,300 WBC/ μ L; 89% mononuclear cells (unimodal), 33% blasts
Dog 9	Mixed Breed	12 y	S		94,400 WBC/ μ L; 75% lymphocytes, 36% blasts
Dog 10	Labrador Retriever	10 y	S	FWBC, DFLT (LM)	47,100 WBC/ μ L; 70% lymphocytes
Dog 11	Golden Retriever	11 y	S		103,000 WBC/ μ L; 70% lymphocytes
Dog 12	Australian Heeler	8 y	S		127,000 WBC/ μ L; 97% lymphocytes
Dog 13	Rottweiler	3 y	S	DFLT (LM)	31,100 WBC/ μ L; 70% lymphocytes
Dog 14	Doberman Pinscher	13 y	S		119,000 WBC/ μ L; 77% mononuclear cells (bimodal), 61% blasts
Dog 15	German Shepherd	9 mo	S		3100 WBC/ μ L; 22% bands, 14% IG
Cat 1	DSH	14 y	S	NRBC, RRBC, DFLT (NLMEB)	WIC:WOC = 3.1; 52% blasts (manual differential)
Cat 2	Mixed Breed	3 y	C	NRBC, DFLT (NLMEB)	WIC:WOC = 9.1; 82% blasts (manual differential)
Cat 3	Mixed Breed	2 y	C	NRBC	WIC:WOC = 4.6; 22% blasts (manual differential)
Cat 4	DSH	5 y	C	NRBC	21,900 WBC/ μ L; 17% bands, 8% IG, 2% blasts
Cat 5	DSH	4 y	S	DFLT (NLMEB)	132 WBC/ μ L; 5% bands, 9% IG, 50% mononuclear cells (unimodal), 2% blasts
Cat 6	Mixed Breed	12 y	C	DFLT (LM)	54,900 WBC/ μ L; 50% lymphocytes

*F indicates female; M, male; S, spayed female; C, castrated male.

†NRBC indicates nucleated RBC; DFLT, default gates for differential (N, neutrophils; L, lymphocytes; M, monocytes; E, eosinophils; B, basophils), NWBC, non-WBC; FWBC, fragile WBC; RRBC, resistant RBC.

‡WIC indicates impedance count of WBCs; WOC, optical count of WBCs; IG, immature granulocytes.

ging diagnostics indicating bands and IG > 40% or IG ≥ 15% of total neutrophils).

3. CBC indicated the presence of >50% lymphocytes, >60% monocytes, or >60% mononuclear cells that distributed as a uniform (unimodal) population that could not be separated into lymphocytes or monocytes.
4. Leukopenia with >10% IG.
5. Mismatch of >2-fold between the WIC and the WOC (WIC:WOC > 2).

Animals for which leukemia was considered in the list of differential diagnoses were considered for the study population. WBC differential counts were confirmed manually for each animal. Table 1 shows the signalment and criteria for inclusion for each animal and the final diagnoses.

Instrumentation

The CD3500 automated hematology analyzer loaded with the veterinary software package (revisions G and H) was used to obtain automated CBC data for each animal.

The CD3500 uses an aperture impedance (von Behrens probe) technique to generate the WIC. For the WOC, blood drawn into the analyzer is diluted with a leukoprotective reagent that maintains WBCs in a condition similar to their natural state and renders RBCs invisible to laser light, such that they do not produce significant laser light scatter ("soft" osmotic lysis).²⁴ The light scatter signatures of approximately 10,000 unstained WBCs are measured and sorted using a multi-angle, polarized, scatter separation technique.²⁶ The angles of light scatter (or transmitted light) used to distinguish individual cellular morphologic characteristics are as follows: 0° (forward angle light scatter) is a relative indicator of cell size, 10° (narrow angle light scatter) is a measure of complexity or nucleus to cytoplasm (N:C) ratio, 90° (right angle light scatter) polarized is equivalent to nuclear lobularity, and 90° depolarized is a gauge of granularity. Data are acquired in list mode, where each cell is identified by number and each characteristic is recorded individually. The optical data are sequentially processed using decision-making algorithms to classify individual cells by their light scatter properties into distinct WBC subpopulations, and from these light scatter signatures a 5-part WBC differential

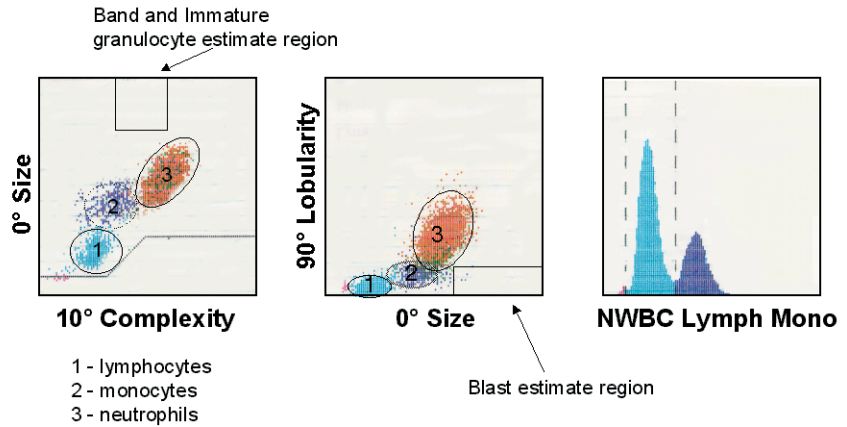


Figure 1. Flow cytometric light scatter profiles of peripheral blood cells from a normal dog. Light scatter plots display blood cell differential information (left and center). Boxes within scatter plots represent regions where abnormal or immature cells tend to localize. The frequency distribution (mononuclear) histogram (right) shows separation of mononuclear WBCs and non-WBC into 3 regions. The first region in the lower channels contains events that do not have light scatter properties consistent with WBCs (NWBCs, including nucleated erythroid cells, platelet clumps, and micromegakaryocytes or giant platelets). The middle region, bordered on each side by floating gates (dashed lines) includes cells with light scatter signatures consistent with lymphocytes, and the third region in the upper channels (to the right of the second gate) includes cells with light scatter signatures consistent with monocytes. The CD3500 printouts do not perfectly align the x-axis legend with the gated regions in the mononuclear histograms.

is generated. The list mode data are configured in x,y scatter plots and subjected to sequential multiparameter analysis.²⁷ Initially, gates are derived from straight lines projected from valleys in scatter plot or histogram data from single parameter data (for example, 0° light scatter vs 10° light scatter). The intersections of these gates create rectangles, and each cell within a rectangle is assigned to a designated classification, which is noted in the list mode table. Each event is further subject to additional analyses (using single or multiple parameters) that may include isometric gating, radial gating, distance gating, and other geometric principles (see Schumacher²⁷ for details). In the generation of a 5-part differential, it is necessary to change or validate a given classification made in 1 dimension based on the result of a later step in another dimension. Changing classifications in process is known as systematic multivariate analysis.²⁷ Nonconforming events that cannot be classified in the 5-part differential create additional opportunities to test for increased heterogeneity or additional subpopulations. For example, immature cells and blasts are generally larger than mature cells; their presence will increase the heterogeneity of a population (with respect to size), and when present in large numbers, these cells can increase the average size of the population. These alterations will generate morphologic flags that can be used to trigger microscopic examination of blood smears or additional tests to classify the abnormal pop-

ulation. Ultimately, each cell counted (event) is represented in light scatter plots by one of the following colors: yellow/orange (neutrophils), blue (lymphocytes), purple (monocytes), green (eosinophils), and gray (basophils). The population distribution data and computerized WIC/WOC algorithm data are used to generate diagnostic "flags" in the presence of abnormal or immature cells and to verify the total WBC count.²⁴

The use of sequential analysis by the CD3500 dictates the use of dynamic gates, ie, gates that are not fixed and limits which can vary somewhat among samples (there is, however, high reproducibility and precision for each sample¹⁹). The analysis first differentiates cells into mononuclear cells (agranular) and polymorphonuclear cells (granular) in isometric histograms of 90° (lobularity) and 10° (complexity) scatter.²⁷ Polymorphonuclear cells are differentiated into eosinophils and neutrophils by radial analysis of 90° depolarized light scatter (granularity) and 90° light scatter (lobularity). Mononuclear cells are further differentiated into lymphocytes, monocytes, intentionally degranulated basophils (due to the effect of the sheath fluid), and non-WBCs (NWBCs) by rectilinear and isometric analysis of 0° (size) and 10° (N:C ratio) scatter. NWBCs, nucleated RBCs (NRBCs), giant platelets, platelet clumps, or hypotonic-resistant RBCs (RRBCs) are gated out of the WBC count and differential.

Blasts are estimated using a region of the 90° (lobularity) and 0° (size) scatter plot that usually contains pleomorphic cells showing moderate to marked anisocytosis with minimal nuclear lobularity. The blast population in this 2-dimensional scatter plot is sometimes colloquially called a flat line. Because the instrument uses dynamic gating, precise limits for this flat line cannot be established, but the presence of cells in this region (Figure 1) should alert the operator to the presence of large cells with no nuclear lobularity that are consistent with blasts. A blast region estimate of >2% (or monocytes >20%) will generate a morphology flag.

IGs, including metamyelocytes, myelocytes, and earlier granulocytic precursors and bands (large polymorphonuclear cells), are estimated based on quadrants built around the neutrophil population in the 0° (size) and 10° (N:C ratio) scatter plots (see Figure 1). If the IG estimate is >3% or the band estimate is >12.5%, or if together they account for >50% of the neutrophils, a morphology flag is generated.

Variant lymphocytes are estimated from the difference between the measured and expected lymphocyte 0° size distributions or the difference between the kinetic-corrected WOC and the WOC. Variant lymphocytes >10% will trigger a morphology flag.

In the analysis of human blood, the blast flag was 81% sensitive and 95% specific, the IG flag was 72% sen-

sitive and 76% specific, and the NRBC flag was 43% sensitive and 94% specific.¹⁹ In addition, the false-negative rate for atypical lymphocytes was only 0.4% when the variant lymphocyte flag in the CD3500 was not activated.¹⁸ The specificity and sensitivity of these morphology flags have not been determined for veterinary species.

The following gain and threshold settings were used for this study. For dogs, gains were 1957 at 0° (forward scatter), 1042 at 10° (narrow angle forward scatter), 1164 at 90° (side scatter), 2444 at 90° depolarized, 2900 for RBC, 2353 for platelets, and 3861 for WIC. The threshold settings were 320 for WBC, 240 for RBC, 396 and 4095 (low and high, respectively) for platelets, and 480 and 4095 (low and high, respectively) for WIC. For cats, gains were 2039 at 0°, 1037 at 10°, 2281 at 90°, 2389 at 90° depolarized, 4095 for RBC, 2468 for platelets, and 3406 for WIC. The threshold settings were 400 for WBC, 240 for RBC, 257 and 4095 (low and high, respectively) for platelets, and 520 and 3000 (low and high, respectively) for WIC.

Scatter plots and frequency distribution histograms for WBC (WOC) generated from a normal canine blood sample are shown in Figure 1 to illustrate the regions where normal and abnormal populations are located.

Classification of leukemic cells

The initial classification of leukemic cells in circulation was based on the CD3500 data (and microscopic confirmation of blasts as indicated below). The sequential algorithms used are shown in Figure 2. Specifically, the first tier of analysis (I) was based on the WIC:WOC ratio. The next tier (II) was based on the presence of granulocytic predominance and/or monocytic predominance with IG and band flags or $\geq 2\%$ blasts. The next tier (III) was based on the presence of leukopenia with blast flags or on the presence of a monomorphic, unimodal population of nongranulocytes. The last tier (IV) included classification of the nongranulocyte population into monocytic (or undifferentiated) or lymphocytic and, for the latter samples, subclassification into acute vs chronic lymphoid leukemia.

Using this algorithm, samples with a WIC:WOC of >2.0 where erythroblasts or megakaryoblasts were identified by microscopic examination would be provisionally classified as acute leukemias with erythroid or megakaryocytic potential (AML-M6Er/AML-M7 by the FAB nomenclature). Samples that had >10% IG with leukopenia and >2% blasts would be provisionally classified as acute myeloid leukemias (AML or nonerythroid, nonlymphoid leukemia, including granulocytic and myelomonocytic subtypes FAB M1-M4). Samples that triggered band and IG flags with leukocytosis (<60,000 WBCs/ μ L, granulocytic predominance) and



Figure 2. Diagnostic algorithm for classification of leukemias based on CD3500 output data. This tiered approach was used to provisionally classify canine and feline leukemias. Band, immature granulocyte (IG), and blast estimates were displayed on the flagging diagnostic screen.

>2% blasts would similarly be classified provisionally as AML. Samples that triggered band and IG flags with leukocytosis (>60,000 WBCs/ μ L, granulocytic predominance) and no blasts would be provisionally classified as chronic myeloid leukemias (CML), although a leukemoid response would have to remain in the list of differential diagnoses for such cases. A preponderance of eosinophils or basophils would be used to further refine the classification of granulocytic leukemias into eosinophilic or basophilic. Samples with leukopenia, no IG flags, and >2% blasts would be provisionally classified as nongranulocytic leukemias. Samples with >60% monocytes and >2% blasts would be provisionally classified as acute monocytic leukemias (AMoL or FAB M5), but undifferentiated leukemias (FAB M0) would probably show similar profiles. Samples with >50% lymphocytes and >2% blasts would be provisionally classified as acute lymphoid leukemias (ALL). Samples with >50% lymphocytes and no blasts would be provisionally classified as chronic lymphocytic leukemias (CLL), although a benign (reactive) lymphocytosis would have to remain in the list of differential diagnoses. Samples with >60% mononuclear cells that comprised a unimodal population that triggered a default flag (inadequate separation into lymphocytes or monocytes) also would be classified provisionally as ALL. Confirmation of these predictions included a review of peripheral blood smears by a pathologist, followed by determination of enzyme cytochemical and immunologic phenotypes (Table 2). Each case of nonlymphoid leukemia was ultimately classified according to the modified FAB criteria proposed by the Animal Leukemia Study Group.^{1,22,28} Lymphoid leukemias were classified as ALL or CLL and modified by their immunologic phenotype (B cell or T cell).

Enzyme cytochemistry and special stains

Demonstration of the activity of chloroacetate esterase (CAE) and N-butyrate esterase (BUE) in blood or bone marrow aspirate smears was performed using commercially available cytochemistry kits (Sigma Chemical Co, St Louis, Mo, USA) according to the manufacturer's recommendations. The specificity of these cytochemical stains (Table 2) was confirmed in the laboratory of one of us (J.W.) and was in agreement with published standards.²⁹ Periodic acid-Schiff (PAS) staining was performed using routine histologic methods.

Immunocytochemistry

Immunocytochemical reactions were performed and validated in the laboratory of one of us (J.W.) as previously described.³ The antibodies used and the respective antigens they recognize are detailed in Table 2. The

specificities of antibodies directed against CD3, CD21, CD79a, Ig, CD45RA, FEr2, CD61, and lysozyme have been published for veterinary species.^{3,11,29-33} Antibodies against CD3, CD79a, cytoplasmic Ig, lysozyme, L1-calprotectin, alpha-1 antitrypsin, vimentin (clone V9), and CD61 were purchased from DAKO (Carpinteria, Calif, USA). Antibodies against CD45RA, CD18, and CD21 were provided by Peter F. Moore (Davis, Calif, USA), and anti-FEr2 was provided by Edward A. Hoover (Fort Collins, Colo, USA).

Indirect flow cytometry

Flow cytometry was performed and validated in the laboratory of one of us (R.S.) as previously described.³ The antibodies used and the respective antigens they recognized are detailed in Table 2. Antibodies to CD45, CD4, and CD8 were purchased from Serotec (Raleigh, NC, USA). Antibodies to CD14 and DR were purchased from VMRD (Pullman, Wash, USA).

DNA content (ploidy) analysis

Leukemic cells were enriched from peripheral blood samples by discontinuous density gradient centrifugation as previously described.³ The proportion of leukemic cells in these preparations was routinely >90% based on the phenotypic criteria. Cells were washed in 150 mM sodium chloride and permeabilized with 70% ethanol overnight at -20°C . The permeabilized cells were washed again in 150 mM sodium chloride, incubated in phosphate-buffered saline containing 0.5% Tween 20 with RNase A (25 $\mu\text{g}/\text{mL}$; Boehringer Mannheim, Indianapolis, Ind, USA) at 37°C for 30 minutes, and stained with propidium iodide (25 $\mu\text{g}/\text{mL}$) for 30 minutes in the dark at room temperature. DNA content of 20,000 cells was evaluated by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif, USA) and the ModFit LT version 2.0 software package (Verity Software, Topsham, Me, USA).

Antigen receptor gene rearrangement

The antigen receptor gene rearrangement assay was developed and validated in the laboratory of one of us (A.C.A.). The assays were performed as described elsewhere.³⁴ (The procedures used to characterize antigen receptor gene rearrangements, including polymerase chain reaction [PCR] amplification of the Ig heavy chain [IgH] V-D-J sequences and of the T-cell receptor gamma [TCR γ] V-J sequences, are currently under patent application.)

Genomic DNA was isolated from enriched

Table 2. Enzymatic and immunologic tests used for phenotyping leukemic cells.

Method	Enzyme or Antigen*	Expression
Enzyme cytochemistry	Chloroacetate esterase	Granulocytes
Enzyme cytochemistry	N-butyrate esterase	Monocytes (diffuse distribution), lymphocytes (focal distribution)
Immunocytochemistry	CD3 ϵ (component of the CD3 signaling complex of the T-cell receptor)	T cells
Immunocytochemistry	CD21 (complement receptor [CR2, C3dR])	B cells
Immunocytochemistry	CD79a (component of the immunoglobulin-signaling complex of the B-cell receptor)	B cells
Immunocytochemistry	clg (component of the B-cell receptor)	B cells (including plasma cells)
Immunocytochemistry	CD45RA (isoform of the CD45 protein tyrosine phosphatases)	Subset of naïve lymphocytes (B cells > T cells)
Immunocytochemistry	CD18 (β integrin)	Panleukocyte marker (in lymphocytes it may be restricted to specific subsets or activated cells)
Immunocytochemistry	Vimentin (V9, intermediate filament)	Mesenchymal cells (presence and subcellular distribution are helpful to establish degree of leukocyte maturation)
Immunocytochemistry	FER2 (antigen with expression restricted to erythroid cells)	Erythroid cells
Immunocytochemistry	Lysozyme	Monocytes; also detectable with lesser intensity in granulocytes when examined by immunocytochemistry
Immunocytochemistry	L1-calprotectin (calcium-binding cytoplasmic protein involved in cellular defense)	Myeloid cells
Immunocytochemistry	Alpha-1 antitrypsin (A1AT)	Monocytes
Immunocytochemistry	CD61 (integrin β 3 subunit)	Mature and immature platelets
Flow cytometry	CD45 ("common leukocyte antigen," isoform of the CD45 protein tyrosine phosphatases)	All WBCs
Immunocytochemistry or flow cytometry	CD4 (adhesion molecule that interacts with MHC class II)	Helper T cells (also neutrophils in the dog)
Immunocytochemistry or flow cytometry	CD8 (adhesion molecule that interacts with MHC class I)	Cytotoxic T cells
Flow cytometry	CD14 (LPS-binding protein)	Monocytes
Flow cytometry	DR (MHC class II antigen)	Professional antigen-presenting cells, including B cells and monocytes
Flow cytometry	slg	B cells

*clg indicates cytoplasmic immunoglobulin; MCH, major histocompatibility complex; LPS, lipopolysaccharide; slg, surface immunoglobulin. Reactivity of anti-FER2 antibody has only been demonstrated in cells of feline origin.

leukemic cells, and IgHV-D-J sequences were amplified using PCR with forward primer Dp1 and reverse primers Dp2 and Dp3. Dp1 is located in framework 3 (FR3) of canine VH genes, and Dp2 and Dp3 are found in two JH gene segments. For PCR, reaction mixtures contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 1% Triton X-100, 1.5 mM MgCl₂, 0.2 mM of each dNTP, and 100 ng genomic DNA. Cycling reactions were performed on a Perkin Elmer System 9600 thermocycler (Applied Biosystems, Foster City, Calif, USA) with an initial denaturation of 30 seconds at 94°C followed by

35-40 cycles of 94°C for 8 seconds, 60°C for 10 seconds, and 72°C for 15 seconds. Ten microliters of the 50- μ L reaction volume was resolved by polyacrylamide gel electrophoresis on 10% gels. TCR γ V-J sequences were amplified with forward primers Dp6 and Dp7 and reverse primers Dp4 and Dp5. Dp6 and Dp7 prime FR3 of V gamma gene segments, and Dp4 and Dp5 are found in two J gamma gene segments. Dp6 is a consensus primer based on human, goat, and bovine sequences. PCR conditions were the same as for IgH amplification.

Cell-Dyn 3500 and Leukemia Lineages

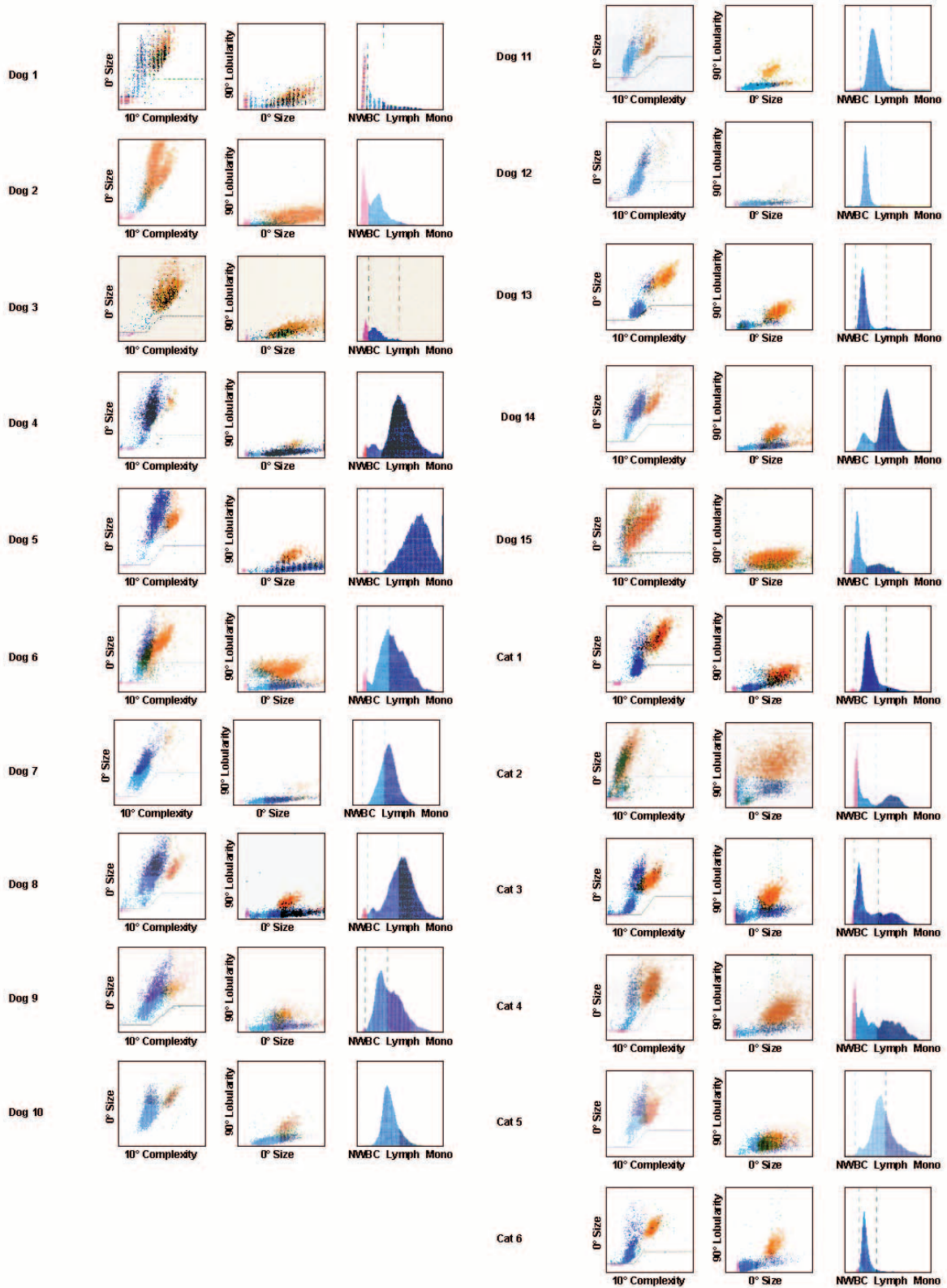


Figure 3. CD3500 light scatter plots and mononuclear histograms of patients with leukemia or leukemic lymphoma. Two-dimensional light scatter dot plots (left: complexity vs size; middle: size vs lobularity) and 1-dimensional frequency distribution (mononuclear) histograms are shown for each animal included in this study (dogs 1-15 and cats 1-6).

Table 3. WBC impedance counts (WIC), WBC optical counts (WOC), and presence of blasts in animals with myeloproliferative disorders with erythroid or megakaryocytic potential.

Animal	WIC (cells/ μ L)	WOC (cells/ μ L)	Diagnostic Criteria (light microscopic observations)
Dog 1	36,000	17,000	Few NRBCs* (17 NRBCs/100 WBCs), predominance of basophilic blasts (megakaryoblasts?), some myeloblasts, poorly differentiated myelomonocytic cells
Cat 1	5600	1800	Moderate number of nucleated erythroid cells recognized as rubriblasts with apparent maturation to rubricytes and occasional metarubricytes (52 NRBCs/100 WBCs)
Cat 2	95,000	10,400	Large number of nucleated erythroid cells, most of which were rubriblasts or prorubricytes, with large number of metarubricytes (75 NRBCs/100 WBCs)
Cat 3	83,800	18,200	Large number of nucleated erythroid cells, most of which were rubriblasts or prorubricytes, with moderate number of metarubricytes (275 NRBCs/100 WBCs)

*NRBC indicates nucleated RBC.

Results

Blood samples collected into evacuated EDTA tubes were analyzed by multiparameter flow cytometry using the CD3500 automated hematology analyzer. Figure 3 shows the CD3500 light scatter plots and mononuclear histograms of the 21 samples examined for this study. None of the samples showed evidence of aneuploidy or other abnormalities of DNA content.

Application of tier I of the algorithm: flags and mismatches between WIC and WOC

A WIC:WOC ratio of >2.0 with NRBC or RRBC flags is suggestive of at least 100 NRBCs/100 WBCs or other NWBCs, such as micromegakaryoblasts, giant platelets, or platelet clumps in the peripheral blood in numbers equal to or greater than those of WBCs. Samples from 14/15 dogs and 3/6 cats had WIC:WOC ratios between 0.91 and 1.1; however, 1/15 canine samples (dog 1) and 3/6 feline samples (cats 1-3) had WIC:WOC ratios of >2.0 , with NRBC flags and blasts in the peripheral blood (Tables 1 and 3). The WIC values were within the reference range for cat 1 (5600 WBCs/ μ L) and were increased for dog 1 (36,000 WBCs/ μ L), cat 2 (95,000 WBCs/ μ L), and cat 3 (83,800 WBCs/ μ L). The WOC values were within the reference range for dog 1 (17,000 WBCs/ μ L), cat 2 (10,400 WBCs/ μ L), and cat 3 (18,200 WBCs/ μ L), whereas cat 1 was leukopenic (1800 WBCs/ μ L). For dog 1, cat 1, and cat 2, the automated WBC differentials resulted in default flags, indicating that the WBCs could not be separated into predetermined subpopulations dictated by the constraints of the 5-part differential settings. For these animals, the instrument reported differential WBC counts based on an idealized 5-part differential based on the preset gains and thresholds for each species. Very few cells from each of these animals segregated to the blast estimate region (see Figures 1 and 3).

Light microscopic examination of peripheral blood confirmed that there were large numbers of NRBCs (including metarubricytes and rubricytes) with variable proportions of blasts that resembled erythroblasts or megakaryoblasts (Tables 1 and 3), indicating that these animals would fit a provisional classification of erythroid or megakaryocytic leukemia (FAB M6Er or M7, Table 4). A proportion of the blasts from each of these cases seemed to distribute into the monocyte region. For example, in dog 1, the CD3500 differential reported 16% monocytes, 1% blasts, and 7 NRBCs/100 WBCs. Yet, examination of peripheral blood smears showed a neutrophilic leukocytosis with a left shift back to metamyelocytes, with monocytes accounting for only 5% of the cells. Conversely, 13% of the nucleated cells were large mononuclear blasts (5-25 μ m in diameter) with round to oval to occasionally indented nuclei, smudged to coarsely granular chromatin, and scant to moderate dark blue, finely granular cytoplasm with frequent cytoplasmic twigs or a few blebs. The observed frequency of NRBCs (erythroid precursors, mostly metarubricytes) was 17/100 WBCs. Macroplatelets (5-10 μ m in diameter) with cytoplasmic features similar to those of the blasts also were observed.

The possible distribution of blasts to the monocyte region in the cats was more variable. For example, the CD3500 WBC differential count for cat 3 included 33% monocytes, and the blast flag also reported 33% cells. The monocyte and blast estimates derived from microscopic examination of peripheral blood smears were 18% and 22%, respectively. The CD3500 WBC differential count for cat 2 included 23% monocytes, and the blast flag also reported 23% cells. However, in this cat the monocyte and blast estimates derived from microscopic examination of peripheral blood smears were 3% and 82%, respectively (Figure 4). The results for cat 1 were even more remarkable, in that the CD3500 WBC differential count included 17% monocytes, and the

Cell-Dyn 3500 and Leukemia Lineages

Table 4. Preliminary and final classification of leukemias.*

Animal	CD3500 Prediction	Final Diagnosis	Phenotype Results (% blasts)†	
			Positive	Negative
Dog 1	AML with erythroid or megakaryocytic potential (M6/M7)	Multipotential leukemia	CAE (weak, 30-50% of cells), BUE (weak, 30-50%), PAS (weak, 10-20%), sideroblasts (<10%), V9 (30-50%), L1 (30-50%), CD61 (15-20%)	CD18
Dog 2	AML	AMMoL (M4)	CAE, BUE (diffuse), L1	ND
Dog 3	CML	CML	CAE (segs, bands, metas, myelocytes, progran, rare MNC), BUE (diffuse; MNC) V9 (variable), CD18, CD45, L1	CD45RA, slg, CD21, CD3, DR, CD14
Dog 4	AMoL	?AMoL (M5a)	BUE (punctate to diffuse), V9, CD18, CD45, DR	CAE, L1, slg, CD79a, CD21, CD3, CD4, CD8, IgH, TCR γ
Dog 5	AMoL	AMoL (M5a)	BUE (diffuse), CD18, CD45, L1, DR, CD14, lysozyme, A1AT	CAE, CD45RA, slg, CD3
Dog 6	ALL	LPD Stage V LSA (B cell)	CD45, CD45RA, slg, CD79a, IgH	CD3, TCRg
Dog 7	ALL	ALL (B cell)	BUE (punctate), CD45, CD45RA, slg, CD79a, IgH	CAE, CD3, TCRg
Dog 8	ALL	ALL (T cell)	BUE (punctate), V9, CD18, CD45, CD3, TCR γ	CAE, slg, CD21, DR, CD14, IgH
Dog 9	ALL	ALL (T cell)	BUE (punctate), V9, CD18, CD45, CD45RA, CD3, CD8	CAE, CD79a, CD21
Dog 10	CLL	CLL (T cell)	BUE (punctate), CD45RA, CD3, TCR γ	CD79a, CD21, CD4, CD8, IgH
Dog 11	CLL	ALL (B cell)	CD79a	CD3
Dog 12	CLL	CLL (B cell)	CD45RA, slg, CD79a	CD3
Dog 13	CLL	CLL (T cell)	BUE (punctate), CD45RA, CD3, CD8, TCR γ	V9, CD18, CD21, CD4, IgH
Dog 14	ALL	ALL (B cell)	BUE (punctate), CD45RA, CD79a	slg, clg, CD3, IgH, TCR;gg
Dog 15	?Degenerate left shift or aleukemic leukemia	LPD Stage V LSA (B cell)	BUE (punctate), slg, CD79a	CD3
Cat 1	AML with erythroid or megakaryocytic potential (M6/M7)	AML (M6Er)	FEr2	CD79a, CD3
Cat 2	AML with erythroid or megakaryocytic potential (M6/M7)	AML (M6Er)	FEr2	CD79a, CD3
Cat 3	AML with erythroid or megakaryocytic potential (M6/M7)	AML (M6Er)	FEr2	CD79a, CD3
Cat 4	AML (nonlymphoid)	AMMoL (M4)	CAE, BUE (diffuse), L1	CD79a, CD3, FEr2
Cat 5	ALL	ALL (T cell)	BUE (punctate), CD3	slg, CD79a
Cat 6	CLL	CLL (T cell)	BUE (punctate), V9, CD18, CD3	CD21, FEr2

*AML indicates acute myelogenous leukemia; AMMoL, acute myelomonocytic leukemia; CML, chronic myelogenous leukemia; AMoL, acute monocytic leukemia; ALL, acute lymphocytic leukemia; LPD, lymphoproliferative disorder; LSA, lymphosarcoma; CLL, chronic lymphocytic leukemia; AML (M6Er), acute myeloid leukemia with erythroid predominance.

†CAE indicates chloroacetate esterase; BUE, butyrate esterase; PAS, periodic acid-Schiff; V9, vimentin (V-9 clone); L1, L1-calprotectin; ND, none determined; segs, segmented neutrophils; bands, band neutrophils; metas, metamyelocytes; progran, progranulocytes; MNC, mononuclear cells; slg, surface immunoglobulin; IgH, monoclonal rearrangement of immunoglobulin heavy chain; TCR γ , monoclonal rearrangement of T-cell receptor gamma; A1AT, alpha-1 antitrypsin; clg, cytoplasmic immunoglobulin.

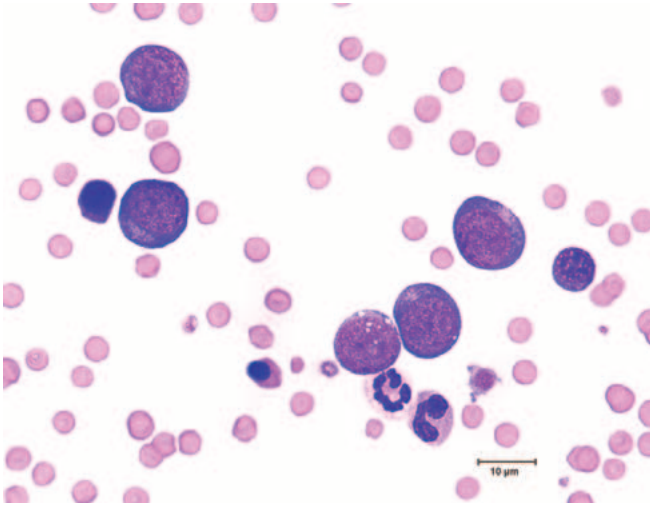


Figure 4. Rubriblasts in peripheral blood from cat 2. Most of the nucleated cells are rubriblasts, with few rubricytes, rare metarubricytes, and occasional WBCs. Wright's-Giemsa, bar = 10 μ m.

blast flag reported only 1% cells. However, the monocyte and blast estimates derived from microscopic examination of peripheral blood smears were 16% and 52%, respectively. For cats 1 and 2, the large discrepancy between the proportions of blasts estimated by the CD3500 and by microscopic examination could only partially be reconciled by a possible classification of blasts as lymphocytes. For cat 2, the CD3500 WBC differential count included 16% lymphocytes, and the estimate derived from microscopic examination was 3%. For cat 1, the CD3500 WBC differential count included 36% lymphocytes, and the estimate derived from microscopic examination was 24%.

The final classification for each of the animals was obtained through additional tests. For dog 1, necropsy findings showed diffuse invasion of leukemic cells in the lymph nodes, spleen, kidneys, liver, heart muscle, and bone marrow and in the oral lesions. Most of the leukemic cells were poorly differentiated, but in some tissues there was progression along the myeloid lineages. Enzyme cytochemistry showed weak reactivity with CAE, BUE (punctate to diffuse), and PAS in 10-50% of the cells; rare sideroblasts were seen in the bone marrow (Table 4). Approximately 30-50% of the cells stained positive for vimentin and L1-calprotectin, and 15-20% stained positive for CD61. Together, the data suggest that this leukemia retained the potential for differentiation along multiple lineages, probably including myeloid, erythroid, and megakaryocytic lineages. The ontogeny of the blasts for each of the cats was confirmed by positive staining with the FEr2 antibody (Table 4); Cats 1 and 2 also tested positive for circulating feline leukemia virus (FeLV) antigen. Thus, the final classifica-

tion for the leukemias in cats 1, 2, and 3 was erythremic myelosis (FAB M6Er).

Application of tier II of the algorithm:
flags and granulocytic leukocytosis

There were 17 animals without a WIC-WOC mismatch. Leukocytosis was noted in 16 of 17 animals; 1 was leukopenic. Among this group of 17 animals, 3 (dog 2, dog 3, and cat 4) had granulocytic leukocytosis with band + IG \pm blast flags that led to further evaluation (Figure 2). In dog 2, there was a bimodal population of "neutrophils" that dominated the $0^\circ/10^\circ$ scatter plot. There was a distinct separation of the granulocytic cells that fulfilled light scatter criteria for bands and IGs (17% and 36%, respectively; Table 1 and Figure 3, $0^\circ/10^\circ$ scatter plots) into an area that occupied the region on the scatter plot where monocytes and monoblasts would be expected to distribute, indicating that these IGs had reduced complexity (higher N:C ratio) and increased size (possibly including a few large granulocytic blasts). The remaining granulocytic cells were classified as mature segmented neutrophils. In the $90^\circ/0^\circ$ scatter plot, the immature granulocyte population expanded towards the region where monocytes normally distribute and extended along the x -axis, with IGs partitioning to the blast estimate region (Figure 1). Despite their size and reduced nuclear lobularity that would have made them morphologically similar to monoblasts, the neoplastic granulocytes were excluded from the mononuclear histogram after the first step in the decision-making algorithm. The provisional classification for this leukemia was AML. Light microscopic analysis of peripheral blood from dog 2 confirmed the presence of approximately 19% blasts that resembled immature myeloid cells and could not be classified simply by examination of Wright's-stained smears. Cytochemical and immunocytochemical analysis showed that the blasts retained expression of both granulocytic and monocytic markers (L1, CAE, and BUE), supporting a final diagnosis of acute myelomonocytic leukemia (AMMoL, FAB M4; Table 4).

Dog 3 differed from dog 2 in that the $0^\circ/10^\circ$ scatter plot was dominated by a single population that extended from the neutrophil distribution region upward and to the left into the blast and IG estimate region (band flag estimate = 23%, IG flag estimate = 18%; Table 1 and Figure 3). The $90^\circ/0^\circ$ scatter plot showed an expansion of the granulocytes into the monocyte region expanding away from the blast estimate region. The frequency and distribution of the cells in the mononuclear histogram confirmed that this granulocytic population was excluded from this analysis. The provisional classification for this leukemia was CML, although a severe, inflammato-

ry neutrophilia with marked left shift (leukemoid response) could not be ruled out at this point. Microscopic examination of blood smears from this dog showed marked neutrophilia with a left shift back to progranulocytes; no blasts were flagged by the CD3500 nor were any seen when blood smears were examined microscopically.

Cat 4 had mild granulocytic leukocytosis (21,900 WBCs/ μ L) with 17% bands, 8% IGs, and 2% blasts. The scatter plots showed cells in the granulocyte population that exhibited a slight decrease in complexity (higher N:C ratio) with a progression of cells that showed increasing size and reduced to absent lobularity (a flat line in the blast cell region of the 90°/0° scatter plots). These cells were mostly classified as monocytes, with only a few granulocytes included in the blast estimate region. These data did not allow discrimination of the malignant cell population beyond a classification of possible AML (nonerythroid, nonlymphoid leukemia; Table 4). The left shift was confirmed microscopically, but there was no orderly progression; approximately 8% of the circulating cells were identified as blasts. There was no evidence of infection with FeLV. The phenotype of the blasts supported a diagnosis of AML (FAB M4; Table 4), and a population of blasts with the same phenotype effaced the bone marrow.

Following the algorithm, Dog 15 was classified in this tier because it was leukopenic (3100 WBCs/ μ L) and had 14% IGs (also 22% bands) and no blasts, suggesting there was a degenerative left shift (consumptive inflammatory disease) or bone marrow injury (possibly due to aleukemic leukemia). The scatter plots from this dog did not allow discrimination of a malignant cell population, although a prominent population of mixed cells seemed to occupy the blast estimate region. This differential diagnosis triggered microscopic examination and additional tests. The left shift was not pronounced on microscopic examination (5% bands and no metamyelocytes), but approximately 10% of the circulating cells were identified as blasts. Immunophenotype analysis showed that the blasts were B cells. These cells effaced the peripheral lymph nodes and the bone marrow, supporting a final diagnosis of stage V lymphoma with circulating malignant lymphocytes.

Application of tier IV of the algorithm:
flags and mononuclear leukocytosis

There were no animals that had leukopenia without immature granulocytes but with blasts (tier III), so the remaining animals were classified according to tier IV of the algorithm. Among these animals, 12 (dogs 4-13, cats 5 and 6) had leukocytosis with dominant populations consisting of relatively homogeneous (unimodal size

distributions) mononuclear cells (monocytic and/or lymphoid). Three patterns were identified among these animals. The first was illustrated by dogs 4 and 5, which showed marked monocytosis with large numbers of blasts (77% and 71%, respectively) and relatively smaller numbers of other WBCs (Table 1 and Figure 3). The pattern of distribution in the scatter plots for both of these dogs showed the monocyte/blast population consisted of variable but generally large cells with minimal complexity (high N:C ratio) and minimal nuclear lobularity; the leukemic cells from dog 4 were smaller than those from dog 5. Band and IG flags were 0% and 1%, respectively, for dog 4 and 1% and 6% for dog 5. Based on these results, the leukemias from dogs 4 and 5 were provisionally classified as AMoL (FAB M5), although the possibility that these cells might represent large undifferentiated blasts (FAB M0) had to be considered. Microscopic examination documented the presence of 74% and 55% cells that resembled monoblasts in the blood of dogs 4 and 5, respectively. Phenotyping results indicated that the cells from dog 4 were poorly differentiated but had evidence of commitment to the monocytic lineage (FAB M5a; Table 4). The phenotypic characteristics of the malignant cell population from dog 5 supported a final diagnosis of acute monoblastic leukemia (FAB M5a; Table 4).³

The second pattern was illustrated by dogs 6-9 and cat 5, which showed leukocytosis characterized by dominant populations of mononuclear cells that could not be separated into discrete lymphoid and monocytic populations by the CD3500 multiparameter analysis (Figure 3). Careful examination of the data for dogs 6, 8, and 9 showed that the separation used to generate a 5-part differential was artificial, based at least in part on the presence of "shoulders" in the forward scatter histograms. The 90°/0° scatter plots from dogs 6-9 and cat 5 each showed an expanded population of mononuclear cells of various sizes with minimal nuclear lobularity that extended into the blast estimate region. The variant lymphocytes flag and the blast flag indicated 30% atypical lymphocytes and 20% blasts in dog 6, 2% atypical lymphocytes and 49% blasts in dog 7, 40% atypical lymphocytes and 33% blasts in dog 8, 9% atypical lymphocytes and 36% blasts in dog 9, and 2% atypical lymphocytes and 2% blasts in cat 5. There was little correlation between the predicted number of variant lymphocytes and the number of cells classified as monocytes in these samples, but the number of blasts and the number of monocytes were identical for dogs 6, 7, and 8. The 5-part differential in dog 9 included only 6% monocytes (probably representing the small population in the far right shoulder that could be discerned in the mononuclear histogram). Conversely, the 5-part differential in cat 5 included 18% monocytes. These cases most likely repre-

sented lymphoid leukemias because (1) lymphoid cells are more plastic than monocytes, and thus lymphocytes can resemble monocytes more commonly than is true for the converse, and (2) blasts could account for some or all of the cells in the monocyte region. Also, the presence of blasts and the large cell size would predict that these conditions were more likely to represent acute lymphoid leukemias than chronic lymphoid leukemias. Based on these features, the leukemias in dogs 6-9 and in cat 5 were provisionally classified as ALL. Microscopic examination of blood from these animals showed that each had cells that were consistent with large lymphocytes and lymphoblasts. The monocytes and blasts estimated from the manual differential counts for dogs 6, 7, and 8 were 0% and 52%, 0% and 93%, and 1% and 50%, respectively, indicating that the CD3500 classification overestimated the monocytes and underestimated the blasts (which were probably the same events) in these three dogs. The monocytes and blasts estimated from the manual differential counts for dog 9 and cat 5 were 5% and 30%, and 13% and 9%, respectively, indicating that the CD3500 classification provided a reasonable separation between monocytes and blasts (which were probably distinct events) in these two animals. Immunophenotyping of the blast cell population and analysis of antigen receptor gene rearrangements in dog 6 confirmed a lymphoproliferative disorder of B-cell origin (Table 4). In addition to peripheral blood and bone marrow involvement, this dog had severe generalized lymphadenopathy, with all normal lymphoid elements effaced by large lymphoblasts with the same phenotype, supporting a diagnosis of stage V lymphoma with blasts in the circulation. The immunophenotype and molecular analysis of cells from dog 7 showed that these were B cells (Table 4), but infiltration of leukemic cells was limited to the spleen and liver with minimal lymphadenopathy, supporting a final diagnosis of B-cell ALL. The immunophenotype and molecular analysis of cells from dogs 8 and 9 and cat 5 showed that these were T cells and supported a final diagnosis of T-cell ALL (Table 4). Cat 5 had no evidence of FeLV infection.

The third pattern was illustrated by dogs 10-13 and cat 6, which showed lymphocytosis (>50%) with no blasts (Table 1 and Figure 3). The pattern of distribution in the scatter plots for each of these animals showed that the lymphocyte population consisted of variable but generally small to medium cells with minimal complexity (high N:C ratio) and minimal nuclear lobularity. The variant lymphocyte flag indicated 16% atypical lymphocytes in dog 10, 19% atypical lymphocytes in dog 11, 75% atypical lymphocytes in dog 12, 3% atypical lymphocytes in dog 13, and 3% atypical lymphocytes in cat 6. None of the results triggered the blast flag. Based on the leukocytosis with elevated numbers of small lympho-

cytes (>50%) and no evidence of circulating blasts, the conditions in dogs 10-13 and in cat 6 were provisionally classified as CLL, although reactive lymphocytosis could not be excluded without additional information. Dog 10 was unique among this group in that there was a small shoulder in the mononuclear histogram, indicating that some cells in this population more closely resembled monocytes (Figure 3). Microscopic analysis of blood smears from dog 10 showed that the predominant cells were small lymphocytes, the monocyte estimates obtained from the CD3500 (8%) and from the manual differential (6%) were in close agreement, and no blasts were seen. Immunophenotyping and analysis of antigen receptor gene rearrangements indicated that this dog had a clonal population of T cells, supporting a final diagnosis of CLL (Table 4). In contrast, microscopic analysis of blood smears from dog 11 showed that most cells were small to medium lymphoblasts, and their immunophenotype was consistent with B cells; thus, the final diagnosis was changed to ALL. In this dog, the provisional diagnosis correctly classified the cells into the lymphocytic lineage but incorrectly subclassified the disease as CLL. Microscopic analysis of blood smears from dog 12 showed that the sample consisted almost entirely (99%) of small lymphocytes that proved to be exclusively B cells upon phenotyping, supporting a final diagnosis of CLL (Table 4). Microscopic analysis of blood smears from dog 13 showed that most cells (76%) were small lymphocytes that were of exclusively T-cell origin, based on phenotyping and analysis of antigen receptor rearrangement, supporting a final diagnosis of CLL (Table 4). Microscopic analysis of blood smears from cat 6 showed that approximately 46% of the cells were small lymphocytes that were of exclusively T-cell origin upon phenotyping, supporting a final diagnosis of CLL (Table 4). This cat also had no evidence of FeLV infection.

Dog 14 had leukocytosis with cells that showed a distinct pattern of light scatter with a bimodal distribution on the mononuclear histogram that reversed the normal pattern (compare Figure 1 and Figure 3). The peak of the larger population was near the expected peak for normal monocytes; however, the distribution for monocytic leukemia with a WBC count of 119,000 WBCs/ μ L would be expected to reflect larger cells, as was the case for dogs 4 and 5. Careful analysis of the flagging diagnostics showed 2% atypical lymphocytes and 61% blasts. As for dogs 6-8, the monocyte estimate (60%) was practically identical to the blast flag estimate in this dog, which presented a new dilemma. However, as for dogs 6-9 and cat 5, when we considered that blasts accounted for all the cells identified as monocytes along with the plasticity of lymphoblasts, the disease in this dog was provisionally classified as ALL. Microscopic analysis of blood smears from dog 14 showed that 22%

of the cells were small to medium lymphocytes and lymphoblasts, and 51% were large blasts that were difficult to assign to a lineage. Enzyme cytochemistry and immunophenotyping for dog 14 indicated that both the lymphocytes and the blasts were B cells, supporting a final diagnosis of ALL (Table 4).

Discussion

Until recently, peripheral blood samples were manually evaluated, and automated methods were not considered practical or dependable when applied to veterinary species. There is mounting evidence that the analysis of multidimensional light scatter data, beyond the 5-part differential, can enhance morphologic observations and add information important for the identification and classification of hematologic disorders.^{3,12,35}

For this study, we constructed a diagnostic algorithm based on output data from the CD3500 and assessed its diagnostic utility to predict the ontogeny of leukemic cells in canine and feline blood. Our results show that the collective properties of the nucleated cell populations in peripheral blood reflected the intrinsic morphologic characteristics of individual cells, whether the cells were normal or abnormal. Moreover, as has been shown previously for human patients,³⁶ the phenotype of blasts in peripheral blood was similar to that of bone marrow blasts in each animal in which it was evaluated by morphologic features, cytochemistry, and immunophenotyping. Several researchers have demonstrated the utility of immunophenotyping by indirect flow cytometry in the classification of acute leukemias,^{8,9,16,17,37} however, few have evaluated the significance of flow cytometric light scatter properties in this regard.¹³ Light scatter measurements have been used as a discriminator to select a population to analyze the expression of cell surface antigens.³⁸ Although light scatter properties have been previously used to separate lymphoid and nonlymphoid leukemias, they have not been used extensively to diagnose acute leukemias with erythroid and megakaryocytic potential.³⁹

Our analysis of CD3500 data from healthy dogs and cats indicated that the WIC and WOC estimates generally showed <9% variation (range 0–9%). Conversely, samples from animals that had WIC:WOC mismatches (higher WIC) generally activated the NRBC, RRBC, or NWBC flags. When flags are generated by RRBCs, evaluation of the sample with an extended lyse protocol will normalize the WIC:WOC. In the laboratory, every sample that was flagged after using the extended lyse protocol triggered microscopic examination. However, for this study we assigned a WIC:WOC ratio of >2.0 as the first limit for inclusion based on comparison of WIC:WOC ratios of bone marrow samples from healthy

dogs (data not shown). In these normal bone marrow samples, the quotient of the WOC divided by the WIC minus the WOC ($WOC/[WIC - WOC]$) approximated the myeloid:erythroid ratio as long as the samples were within the linear range for the instrument ($\leq 200,000$ events). This observation suggested that some nucleated erythroid precursors can be masked from the optical detector because their light scatter signatures may be more similar to those of NRBCs (or other NWBCs) than to those of mononuclear WBCs. The data from cats 1 and 2 add credence to this conclusion because the discrepancy between the WIC (which measures all nucleated cells based on impedance) and the WOC (which measures light scatter properties of WBCs) could not be explained by the misidentification of blast cells (of erythroid lineage) as mononuclear cells (monocytes and lymphocytes). In contrast, the blasts in the circulation of dog 1 and cat 3 might have been identified as monocytes (without activating the blast flag). It is unclear how the immature nucleated erythroid cells that are masked from the laser light might differ from those cells that are classified as mononuclear cells; we believe this observation merits confirmation and further investigation.

Overall, the algorithm was useful to predict the lineage (erythroid/megakaryocytic, AML, AMoL, CML, lymphoid) in 19 of 21 animals (all except dogs 1 and 15) when the final diagnosis was confirmed using the criteria established by the Animal Leukemia Study Group classification scheme (modified FAB)²⁸ for nonlymphoid leukemias and using cytochemical and immunologic results for lymphoid leukemias (Table 4). The results are tempered by the small sample size, the overrepresentation of lymphoid leukemias, and the occurrence of only 2 cases of AML (both M4), 2 cases of AMoL, and a single case of CML. No cases representing FAB M0, M1, M2, or M3 were identified during the period of study, precluding the applicability of the algorithm to these diseases. After this study was closed, the dependability of the algorithm was confirmed in the diagnosis of 9 additional canine leukemias (7 at Texas A&M University and 2 at the University of Florida) and 2 additional feline leukemias (at Texas A&M University). The canine leukemia cases included AMoL ($n = 1$), AMMoL (M4, $n = 2$), lymphoid leukemia ($n = 5$, 2 ALL and 3 CLL, including one of large granular lymphocytes and a CLL in blast crisis), and acute megakaryoblastic leukemia (FAB M7; CD61-positive blasts in circulation). The feline leukemias included 1 case of lymphoid leukemia (ALL) and 1 case of erythremic myelosis (FAB M6Er). As for dog 1 and cats 1-3, the WIC:WOC in the cases of canine megakaryocytic leukemia and feline erythremic myelosis were >2.0, and some of the leukemic cells appeared to be masked from the WOC. Although these cases help to validate our conclusions, they still did not include

examples of FAB M0, M1, M2, or M3 leukemias, which was not surprising based on the low frequency with which these diseases are seen at veterinary referral institutions.

Although automated WBC differentials can be as accurate or more accurate than manual techniques,⁴⁰ our study also highlights some limitations to these techniques. WBCs differentiated by impedance or flow cytometric techniques represent random events that follow the standard error of proportion; therefore, the more cells counted the more precise the result.⁴¹ In dog 1, there were <5000 WBCs/ μ L (WOC), and the blast flag indicated these cells comprised only 1% of the total nucleated cell population; the scatter plots were similar to those seen with a degenerate left shift, so the provisional classification relied exclusively on other information (WIC:WOC ratio and microscopic identification of blasts). Comparison of the CD3500 data with the manual differential counts revealed that most blasts were misclassified as monocytes in this case. The use of a WIC:WOC of >2.0 as the first tier of discrimination also might bias the classification of multipotential leukemias into the FAB M6 or M7 categories when there are circulating NRBCs and blasts that retain erythrogenic or megakaryocytic potential (as for dog 1), which merits a note of caution. Cat 4 also had <5000 WBCs/ μ L and 2% blasts, and the encroachment into the blast estimate region by an expanded population of variable cell size and minimal nuclear lobularity was helpful to provisionally classify the condition as nonlymphoid (hence AML by exclusion). Therefore, a weakness of the algorithm is that provisional classification of leukemic cells in severely leukopenic animals will carry a degree of uncertainty. In addition, the classification of cells into monocytic leukemias, acute lymphoid leukemias, and chronic lymphoid leukemias in tier IV largely relies on separation of cells based on size (forward light scatter). As was shown for dog 11, this size criterion can lead to misclassification of acute leukemias composed of small blasts into the chronic category, although this pattern may be expected for CLL in blast crisis. Because the prognosis and treatment of acute and chronic lymphoid leukemias are remarkably different, the application of the proposed algorithm to information obtained from the CD3500 is useful only to assign provisional classifications, and each diagnosis should be confirmed by microscopic analysis and additional tests. Nevertheless, even in cases such as dog 11, application of the algorithm could reduce the battery of tests applied after microscopic identification of blasts to those appropriate to classify lymphocytes into prognostic categories (ie, B vs T cells).

Although current recommendations include that the morphology of leukemic blasts be verified before

immunophenotyping,³⁸ distinctive light scatter patterns generated by the CD3500 appear useful for identifying morphologic features, which can minimize the speculative and subjective nature of selecting relevant antibodies without losing diagnostic accuracy. Moreover, the combination of morphologic and immunocytochemical phenotyping can improve reproducibility and accuracy of leukemia classification.⁴²

The CD3500 provides rapid and quantitative results that may improve diagnostic decisions, therapeutic choices, and prognostic accuracy for patients with hematologic malignancies. An algorithm derived from the CD3500 output data was useful for predicting the lineage of at least some types of myeloid and lymphoid leukemias in the peripheral blood of dogs and cats. Additional work is necessary to refine this algorithm and to confirm the sensitivity and specificity of these classifications. Furthermore, evaluation of a more representative sample size is needed to assess the utility of this algorithm across the full spectrum of myeloproliferative and lymphoproliferative disorders. \diamond

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