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Flow Cytometric Assessment of Hematopoietic Neoplasia in the Dog (13-Nov-2004)

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Immunophenotyping refers to the application of antibodies specific for differentiation antigens of leukocytes to identify lineages of these cells present in reactive (inflammatory) or neoplastic diseases involving the immune system. Immunophenotyping is an objective adjunct to conventional morphologic assessment. Immunophenotyping can be performed on fluids (such as blood, bone marrow and tissue suspensions) by flow cytometry. It can also be performed on snap frozen tissue sections, formalin-fixed paraffin sections and unfixed cytological smears and blood smears. Flow cytometric immunophenotyping has the advantages of being a fast, objective, sensitive and quantitative method [1]. There are numerous diseases in which the application of immunophenotyping is a crucial element in the attainment of an accurate diagnosis and therefore the provision of an accurate prognosis or institution of appropriate therapy.

Most immunophenotyping is performed with monoclonal antibodies (Mab). Leukocyte antigen workshops have focused on clustering of Mab with similar patterns of reactivity in diverse cells and tissues. This systematic approach has led to the emergence of a common nomenclature for human antigens and their homologues in other species ("Cluster of Differentiation" or CD antigens). Workshops have been conducted in many veterinary species, including dogs [2]. With few exceptions, Mab specific for leukocyte antigens label cells of diverse lineages. **Therefore, to confidently identify specific cell types in blood or in lesions, it is almost always necessary to consider the results obtained with multiple Mab i.e., a "panel" of antibodies, resulting in detection of a "constellation" of antigen expression.**

Diagnostically Important CD Molecules for the Assessment of Hematopoietic Neoplasia in the Dog

Despite the large number of CD molecules identified, not all are useful in immunodiagnosics. The following glossary consists of the CD and related molecules of most importance in veterinary immunodiagnosics for which species specific Mab are available.

CD1 - There are 5 CD1 genes (A-E); the protein products of these genes are CD1a-e. The classical CD1 molecules consist of CD1a, CD1b and CD1c, which are differentially expressed on leukocyte populations. Canine CD1a, CD1b and CD1c molecules have been characterized [3]. CD1 molecules are distantly related to MHC class I; they present peptide, lipid and glycolipid antigens to T cells. CD1 molecules are expressed by cortical thymocytes, but not by mature T cells. CD1 molecules are the best markers of dendritic antigen presenting cells (APC), although subpopulations of B cells and monocytes express CD1c. The vast majority of histiocytic proliferative disorders in the dog involve dendritic APC and are best defined by expression of CD1 molecules, lack of expression of lineage specific lymphoid markers (CD3 and CD79a), and co-expression of molecules of functional importance to dendritic APC such as CD11c and MHC II [4-6]. Subpopulations of B cells and monocytes can also express CD1c, which is of relevance to the investigation of canine B cell CLL (see below).

T Cell Receptor / CD3 - Antigen recognition by T cells occurs through a surface receptor complex. The T cell receptor (TCR) / CD3 complex is only expressed on the surface of mature T cells (and thymocytes). There are two types of TCR designated as TCR $\alpha\beta$ and TCR $\alpha\delta$. They are heterodimeric molecules and are connected by a disulfide bond. Each of the polypeptide chains in the TCR heterodimer contains a variable region (responsible for binding antigen) and a constant region. The TCR is associated with the CD3 complex (five different polypeptides: CD3 γ , CD3 δ , CD3 ϵ , CD3 ζ , CD3 η), which is the

signal transduction portion of the receptor complex in both TCR types. To facilitate signalling through the T cell receptor complex, T cells also express co-receptor surface proteins, CD4 (helper T cells) and CD8 (cytotoxic T cells), which associate with components of the TCR complex during antigen recognition. Mab specific for the CD3 ϵ component of the CD3 complex in dogs are available. CD3 ϵ expression is largely limited to mature T cells, although activated NK cells can express CD3 ϵ in their cytoplasm. Demonstration of CD3 expression is one of the most useful immunophenotypic analyses currently performed in veterinary immunodiagnostics and is used for the diagnosis of T cell leukemia and T cell lymphoma. CD3 expression, with rare exceptions, confirms the presence of T cells in a lesion, although it does not distinguish $\alpha\beta$ and $\gamma\delta$ T cells. Mab specific for $\alpha\beta$ and $\gamma\delta$ T cells exist in the dog and can be used to further classify the type of T cell proliferation that is present.

B Cell Antigen Receptor / CD79 - The B cell antigen receptor complex (BCR) consists of surface Immunoglobulin (sIg) (which binds antigen) complexed with 2 invariant molecules which function as signal transduction molecules (CD79a, CD79b). CD79 is the B cell equivalent of CD3 in T cells. Other components, which form the BCR co-receptor complex, include CD19, CD81, and 2 complement receptors (CR): CD21 (CR2) and CD35 (CR1). CD79a (MB-1) is expressed throughout all stages of B cell development and persists into the plasma cell stage (despite absent or diminished sIg on plasma cells). CD79a is a useful marker for establishing the diagnosis of B cell lymphoma and leukemia, since it is present in the BCR of all B cells regardless of the isotype of the sIg receptor. Background associated with Ig stains in tissues is also not an issue. CD79a is also useful in the diagnosis of cutaneous plasmacytoma (about 80% have focal to diffuse expression). HM57 Mab (Dako) is specific for a cytoplasmic peptide sequence (of CD79a) that is well conserved in diverse species (human and mouse) and is detectable in formalin fixed tissue sections. HM57 also has good reactivity with B cells in dog, cat and horse, although CD79a sequence is unavailable in these species.

CD4 - In the dog, CD4 is expressed by MHC class II restricted T helper cells. Canine neutrophils constitutively express CD4 and in this respect differ from neutrophils of all other species for which data are available [7]. Monocytes, macrophages and dendritic/APC can upregulate CD4 in some instances.

CD5 - CD5 is expressed on mature T cells, thymocytes, and the B-1 subset of B cells. CD5 is associated with the TCR and BCR. CD5 modulates signalling through the antigen-specific receptor complexes. In peripheral mature T cells, CD5 acts as a co-stimulatory signal receptor. Monoclonal antibodies specific for CD5 are available for many species of veterinary importance, including both dogs and cats. CD5 is a marker of the B-1 subset of B cells which are expanded in B cell chronic lymphocytic leukemia (B-CLL) of humans [8]. CD5 is not expressed in B-CLL in dogs (see below).

CD8 - CD8 is a dimeric molecule that is expressed by MHC class I restricted T cytotoxic cells. T cells usually express CD8 $\alpha\beta$ heterodimers; although they can express CD8 $\alpha\alpha$ homodimers. A subset of natural killer (NK) cells may also express CD8 $\alpha\alpha$ homodimers.

CD11 / CD18 - The β_2 integrins (CD11/CD18) are the major leukocyte adhesion molecule family. The absence of β_2 integrins due to mutations in CD18 results in leukocyte adhesion deficiency syndrome (LAD-I) described in humans, Irish Setter dogs, and Holstein cattle. Most leukocytes express one or more members of this family. CD18 is the β_2 subunit which pairs with one of four α subunits to form a heterodimer. Therefore, positivity for CD18 indicates the presence of the β_2 subunit, but does not indicate which of the 4 integrin molecules is present. The four α subunits are: CD11a (all leukocytes), CD11b (granulocytes, monocytes, some macrophages), CD11c (granulocytes, monocytes, dendritic antigen presenting cells) and CD11d (macrophages and T cells in splenic red pulp, and large granular lymphocytes). Antibodies to canine CD18 and all four α subunits have been developed and characterized [3]. Canine CD18 and CD11d are detectable with Mab in formalin fixed tissue sections as well.

CD21 - CD21 is a complement (C3dg) receptor (CR2) that complexes with components of the B cell antigen receptor complex (sIg, CD79a, CD79b), CD19 and CD35 (CR1). CD21 is expressed on **mature** B cells and follicular dendritic cells of the germinal center. Detection of CD21 is useful in the diagnosis of B cell lymphoma and B cell leukemia.

CD34 - CD34 is a heavily glycosylated surface glycoprotein which is expressed on early lympho-hematopoietic stem and progenitor cells, small vessel endothelial cells, embryonic fibroblasts and bone marrow stromal cells [9]. Monoclonal antibodies specific for CD34 have been described in humans, mice and dogs [9,10]. Ligands for CD34 include L-selectin (CD62-L) and E-selectin (CD62-E). Therefore, CD34 may play a role in leukocyte endothelial interactions. CD34 expression has proven useful in diagnosis of acute, immature cell leukemias of both myeloid and lymphoid types in humans. CD34 is not expressed in CLL, lymphoma or myeloma, which are malignancies of more mature cells. A high proportion of acute myeloid

(AML) and acute lymphoblastic leukemias (ALL) in dogs also express CD34 [11]. In our experience (see below), assessment of CD34 expression in some instances of canine lymphoid leukemia has proven useful in the differentiation of ALL (CD34+) from lymphoma with leukemic involvement (CD34-), or CLL (CD34-) when the diagnosis has been problematic on the basis of history, clinical signs and routine hematologic data and morphology [11].

Leukemia

Background (Lessons from the Human Experience) - In the past, the schemes for the classification of leukemias have traditionally been clinically and cytomorphologically based, supplemented in recent decades by the application of cytochemical techniques [12]. However, since the early eighties, major advances in the areas of immunology (especially monoclonal antibody technology), cytogenetics and molecular genetics have occurred. Immunological and cytogenetic analysis of clones of leukemic cells have resulted in a review, and oftentimes, re-interpretation, of the clinical and hematological features of a given disease. These advances have led to the recognition of many new entities within the major categories of both the acute and chronic leukemias. These entities differ in their biological features, including their prognoses [8,12,13].

The utility of immunophenotypic assessment of hematopoietic neoplasia has now been firmly established in people [8,13]. Immunophenotypic assessment significantly impacts diagnosis, prognosis and therapy. This is because the diagnosis and precise classification of hematopoietic malignancies, both leukemias and lymphomas, by morphologic criteria alone has significant limitations [13]. The basic premise of immunophenotyping is that leukemias and lymphomas are the neoplastic counterparts of subpopulations of normal lymphoid and myeloid cells [14]. In general terms, they tend to maintain a constellation of antigen expression similar to their normal counterparts that reflects both their lineage and stage of maturation arrest and clonal expansion. This paradigm has not only proven very useful in the diagnosis and classification of hematopoietic neoplasia but has also assisted in the determination of normal hematopoietic ontogeny.

Immunophenotyping is one of the primary diagnostic modalities for assessment of acute leukemias in people. It is very helpful in differentiating very primitive or poorly differentiated Acute Myeloid Leukemia (M0 and M1 AML) from Acute Lymphoblastic Leukemia (ALL) and determining the various subtypes of both AML and ALL [8,13-15]. These subtypes have markedly differing prognoses and therapies. It is also very helpful in characterizing, and hence determining the prognosis, of the inevitable acute or blastic stage of chronic myelogenous leukemia in humans. Flow cytometry has assumed a very important role in the detection of minimal residual disease (MRD) in people. In general terms, flow cytometry is far more sensitive than routine morphologic assessment in the detection of MRD. Reported sensitivities of detection are in the range of 0.01 - 1.0 %, depending on the phenotype of the neoplastic cells [8,13]. These studies generally use multiparameter staining to detect tumor specific antigen combinations. Immunophenotyping is also one of the primary diagnostic modalities (along with routine morphology and CBC) for the assessment of chronic lymphoproliferative disorders (CLPD) in people [8,13,14]. Each of the CLPD have typical cytologic features. However, because of overlap of these features, morphology alone is not always sufficient to consistently separate the various entities, which differ markedly in prognosis and therapy. In addition, immunophenotyping is a crucial adjunct in the diagnostic work up of lymphoma in people. It is considered essential for a precise, reproducible and clinically relevant classification of lymphoma [13]. Techniques include routine immunohistochemistry of tissue sections or flow cytometric evaluation of appropriately processed tissue biopsies or aspirates of lymph nodes, other tissues, blood or bone marrow.

For many reasons, immunophenotyping in humans has achieved a much greater level of complexity, sophistication and importance than currently exists in veterinary medicine. Nevertheless, the same principles apply and as this discipline develops in veterinary medicine, it is clear that similar useful information can be derived from immunophenotypic studies in animals. **Immunophenotypic assessment has already proven very useful in the diagnosis of leukemia, lymphoma and cutaneous round cell tumors in dogs** [3,6,11,16-18].

Chronic Lymphocytic Leukemia (CLL) - Canine CLL differs markedly from human CLL. In the largest canine study to date, canine chronic lymphocytic leukemia (CLL) occurred in older dogs (mean age 9.75 years; range 1.5 - 15 years; n=73 cases) [11]. Blood lymphocyte counts ranged from 15,000/ul to 1,600,000/ul. Surprisingly, 73% of CLL cases involved proliferation of T lymphocytes (CD3+), and 54% of CLL cases had granular lymphocyte (GL) morphology [11]. GL CLL's were almost exclusively proliferations of T cells that expressed CD8 and the leukointegrin CD11d and more frequently expressed T cell receptor (TCR) $\alpha\beta$ (69%) than TCR $\gamma\delta$ (31%). Canine GL CLL followed a typically indolent clinical course. The non-GL T cell CLL cases (19% of CLL) involved proliferation of TCR $\alpha\beta$ T cells in which no consistent pattern of CD4 or CD8 expression was found. B cell CLL, based on expression of CD21 (and lack of T cell antigens) or CD79a, accounted for only 26% of canine CLL cases. No cases of canine B cell CLL expressed CD5. CD1c expression was present in 95% of canine B

cell CLL. These results are in marked contrast to people where greater than 95% of CLL's involve proliferation of CD5+ B lymphocytes [8].

Canine B cell CLL appeared to be a primary bone marrow disease. However, bone marrow involvement in T cell CLL involving proliferation of GL's appeared to occur relatively late in the course of the disease [11]. The neoplastic expansion in these instances appeared to originate in the spleen. In several cases of GL CLL, concurrent aspirates of spleen and marrow were available for assessment. In most of these cases, there was marked splenic infiltration with GL's but bone marrow involvement was either minimal or inapparent. Occasionally, there was significant marrow infiltration but this was accompanied by a greater degree of splenic involvement. Interestingly, CD11d expression is relatively constrained in tissue; the splenic red pulp is the dominant site of CD11d expression in the hemopoietic system in both dogs and cats [19].

Acute Leukemia - Similar to the experience in people, immunophenotyping also appears to be useful in the assessment of acute leukemias in dogs. In the aforementioned study of canine CLL, a total of 38 cases of acute leukemia were also assessed and interrogated with a panel of 30 monoclonal antibodies, most of which were canine specific (the small remainder being cross reactive) [11]. The mean age was 7.4 years with a range of 0.5 - 13 years. The leukocyte counts varied from 3,300 - 450,000/ul (3.3 - 450 x 10⁹/L, reference interval 6 - 17 x 10⁹/L) with a mean of approximately 133,000/ul (133x10⁹/L). 54% of cases had counts < 100,000/ul (100 x 10⁹/L) and 23% of cases had counts < 50,000/ul (50 x 10⁹/L). Probable acute myeloid leukemia (AML) accounted for 55% (21/38) of cases, B cell (CD 79a+) acute lymphoblastic leukemia (ALL) accounted for 16% of cases (6/38), acute leukemia of GL type for 18% (7/38) and acute undifferentiated leukemia (AUL) for 11% (4/31). Of the cases of GL acute leukemia, 3 were T cell (CD3+ TCRαβ+ CD8αβ+ CD11d+) and 4 were considered to be most likely Natural Killer (NK) cell (CD3- CD11d+ CD8α+). When it was assessed, CD34 expression was observed in 11/12 cases (92%) of AML , 2/2 cases of B cell ALL and 0/3 cases of GL acute GL leukemia. No cases of CLL (of any subtype) expressed CD34. CD34 expression was not assessed in any of the cases of AUL. Similar to its chronic counterpart, GL acute leukemia in the dog appears to be a primary splenic disease; the lack of CD34 expression in the cases of GL acute leukemia in which it was assessed is also consistent with this conclusion.

We have subsequently assessed CD34 expression in greater numbers and a wider variety of canine hematopoietic neoplasia. We have found lack of CD34 expression in lymphoma (0/299, including 204 B cell lymphomas and 95 T cell lymphomas) and common CD34 expression in acute leukemias (10/10 B-ALL, 2/3 T-ALL, 9/10 AUL and 18/20 AML). As stated previously, assessment of CD34 expression was found to be useful for the differentiation of ALL (CD34+) from some cases of primary lymphoma with marked leukemic involvement (CD34-) and some unusual cases of CLL (CD34-).

While antibodies to CD molecules such as CD11b, CD13, CD14, CD15 and CD33 are considered useful for the immunologic diagnosis of AML in people, these molecules are considered to be lineage associated, and not lineage specific [1]. Myeloperoxidase is currently considered to be the definitive myeloid specific marker [1]. Additionally, immunophenotyping of AML using anti-MPO antibodies is also considered to be more sensitive than cytochemical demonstration of MPO enzymatic activity. The use of antibodies to myeloperoxidase have been shown to have the greatest sensitivity and specificity in the diagnosis of AML in people [20]. Many monoclonal antibodies to human or murine myeloid antigens (ie. CD13, CD33) do not cross react in the dog. Additionally, antibodies versus human MPO also do not cross react in the dog (Vernau and Moore, unpublished observations). Therefore, while lack of lymphoid antigen expression and expression of a constellation of myeloid associated antigens is very supportive of a myeloid origin [11], **definitive** immunophenotypic confirmation of AML in the dog is currently not possible. In an effort to rectify this situation, we have recently developed monoclonal antibodies specific to canine myeloperoxidase (Vernau W., Graham P. and Moore P.F., unpublished). We have currently assessed 34 cases of acute leukemia with these canine specific anti-MPO antibodies. Results thus far indicate that these canine specific MPO Mab are useful for the definitive diagnosis of AML in the dog. Thirteen of seventeen (13/17) canine acute leukemias were classified as AML on the basis of MPO positivity that confirmed their myeloid origin. The four MPO negative canine acute leukemias that were still classified as AML's consisted of 2 Acute Megakaryoblastic Leukemias and 2 probable Acute Monoblastic Leukemias (although most of these were MPO positive). The Acute Megakaryoblastic Leukemias were confirmed on the basis of CD41 (GpIIb) positivity [21].

Many cases of acute leukemia in the dog involve proliferation of cells with a primitive or immature morphology that is unhelpful in predicting possible lineage [11]. Additionally, there appears to be a relatively poor correlation between morphologic appearance of the neoplastic cells and immunophenotype, confirming the necessity of immunophenotyping to determine the origin or lineage of the neoplastic cells. Similar to the situation in people, routine morphologic assessment appears to have significant limitations in the diagnosis of canine acute leukemia [11,13] .

Caveats and Limitations

The same major caveats apply to use of this technology in all species. Correlating the clinical outcome with specific antigens rather than the total phenotype is probably not useful. Additionally, the optimal utilization of immunophenotypic data in patient care should include integration of all historical, clinical, morphologic and other (ie. cytochemical, cytogenetic, molecular genetic) information. Unequivocal confirmation of neoplasia is rarely possible solely by the use of immunophenotyping. While careful assessment of clinical, morphological and immunophenotypic criteria are critical, it is generally accepted that assessment and demonstration of clonality by molecular genetic analysis of antigen receptor genes provides the most objective and accurate predictor of lymphoid neoplasia.

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