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Review Article

APPLICABILITY OF FLOW CYTOMETRY IN IDENTIFYING AND STAGING LYMPHOMA, LEUKEMIA AND MAST CELL TUMORS IN DOGS: AN OVERVIEW

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Abstract: Lymphomas, leukemias and mast cell tumors belong to the most important group among all neoplasms affecting dog species. Diagnosis, staging and determining the cell type involved in a specific tumor represent a challenge for researchers and clinicians, and plays a crucial role in treatment efficacy and prognostic purposes. Many different gold standard techniques such as cytology, histopathology, immunohistochemistry and cytochemistry are used to routinely diagnose and stage these tumors. In the recent years flow cytometry is becoming more applicable in veterinary medicine since a wide number of health conditions can be analyzed in a short period of time with a high accuracy. Multiparametric analysis performed by flow cytometry is considered as one of the main advantages of this technique since cell populations can be analyzed for different superficial markers at the same time. Immunophenotyping and staging of tumor cell populations performed by flow cytometry can help in reaching a confirmatory diagnosis and appropriate prognosis of the disease. Moreover, many flow cytometric results have been linked to a high prognostic relevance especially in neoplastic disorders. However, flow cytometry results are compatible and should be interpreted in compliance with data obtained by histopathology, immunohistochemistry and cytology.

Key words: flow cytometry; antibodies; diagnosis; lymphoma; leukemia

Introduction

Neoplastic disorders are considered as one of the most frequent pathologies not only in human medicine, but also veterinary medicine as well. Neoplastic conditions may affect dogs just as all other animals (1).

The term lymphoma often refers to a type of neoplasia where lymphocytes are strictly involved. The updated Kiel Classification is commonly used to classify lymphomas in two categories based on their grade of malignancy, as Low and High grade (2, 3). Further, histopathology is recommended to determine the neoplastic entity of the tumor. The classification proposed by Valli and colleagues (4) classifies dog lymphoma in six most common entities: diffuse large B cell lymphoma (DLBCL), marginal cell lymphoma (MZL), peripheral T cell lymphoma, T-zone lymphomas, T cell lymphoblastic lymphoma, and follicular lymphoma.

Different categories of dog lymphomas have great similarities to human and mouse lymphomas as well. Actually, a study performed by Morse and colleagues have compared similarities between mice and human lymphoid neoplasms (5). This study has also made a classification of mice lymphoid neoplasms following the World Health Organisation (WHO) classification using an appropriate terminology. Another study has classified the non-lymphoid neoplasms of mice aiming to create a consensus among clinical pathologists regarding this issue (6).

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After diagnosis has been established, staging and prognostic features of the disease in peripheral blood, bone marrow, spleen and liver using cytology, western blotting, histopathology, immunohystochemistry and immunocytochemistry should be performed in order to design an adequate therapy protocol (7). Moreover, it has been showed that immunohystochemistry can be used for the identification of lymphoid cells in normal or inflammatory conditions (8)

Among leukemias, three main types are distinguished; acute, chronic and lymphoma with a leukemic phase. Acute leukemias usually arise in bone marrow due to genetic mutations preventing the cells to maturate and proliferate, and are classified as lymphoid, myeloid or undifferentiated. Most acute leukemias in dogs have myeloid origin (9). Diagnosis of acute leukemia is based in finding more than 20-25% bone marrow infiltration from blasts (10). On the contrary, chronic leukemia's are characterized by a highly abnormal presence of mature cells in the peripheral blood (11). Chronic leukemia's can have lymphoid or myeloid origin and diagnosis for both is difficult since it is based on clinical sings and mostly by excluding other pathologies that can affect the number of mature cells in the peripheral blood circulation.

Mast cell tumors (MCTs) are among the most frequent skin tumors in dogs with an overall incidence of 7-25% (12). The cytological evaluation is recommended as a first approach since it can reach to a diagnosis on 96% of cases when a MCT is suspected (13). After cytology, the histopathologic evaluation is needed to confirm diagnosis and to define the grade of the MCT. Two main systems define the tumor grade: a) Patnaik, classifying the tumor in three grades (I, II, III); and b) Kiupel, classifying tumors in high and low grade (14, 15). Further, immunohistochemistry can be of great relevance when evaluating prognostic biomarkers such as Ki67 and CD117 (16, 17). Staging of MCTs can be performed by different techniques but the histological and cytological examination of lymph nodes (18), spleen and liver, and cytological examination of bone marrow and peripheral blood are the main procedures used to identify mast cells infiltration. However, staging of MCTs is very challenging since mast cells can be found in different tissues also in normal or reactive conditions (19).

Other than indicating the many clinical values of Flow Cytometry (FC) in diagnosing and staging lymphomas, leukemias and MCTs, one of the main aims of this review is to promote this relatively new technique for the western Balkan region.

Flow Cytometry

Flow cytometry technique discovered in the sixties, allows the measurement of physical and fluorescent characteristics of separated cells or any other particle, such as nuclei chromosome preparations, microorganisms in a suspension, passing through a light source. There are two different types of flow cytometry, sorting and non sorting. The difference between these types is that with the sorting one, the technician has the possibility to sort different population of cells or particles (20). This type of flow cytometer provides the opportunity to analyze a specific cell population which can be of major interest by identifying its composition. Briefly, a flow cytometer is composed by the sample chamber, a sample stream, a photodetector system and one or more lasers (21). Cells in saline suspension pass through a laser beam in a single row.

The light passing through a single cell is refracted and diffracted. This information is captured and converted in scatter/dot plots providing information on cell size and complexity (Figure 1).

When cells are labeled, the laser beam excites the fluorochromes conjugated to the antibodies giving a positive signal expressed in percentages. Moreover, multiparametric analysis can be performed obtaining data of interest on cell populations for different antigen expression at the same time (22).

As in other tests it is important to run negative control sample to avoid false positive signals, incorrectly considering a cell population as positive. In most cases isotype controls are used, but also a negative cell population to a specific antibody (ex: T-Lymphocytes to CD21) can be adequate for this purpose. Fluorescence minus one (FMO) tubes should be performed when a new flow cytometric experiment is taking place in order to set the correct instrument compensation (23).

Other than immunophenotyping, flow cytometry technique can be used for a wide variation of analysis such as; Minimal Residual Disease, DNA content, apoptosis, proliferation markers (ex. Ki67), immunoresponse to vaccines, external cell activities such as in allergies, etc.

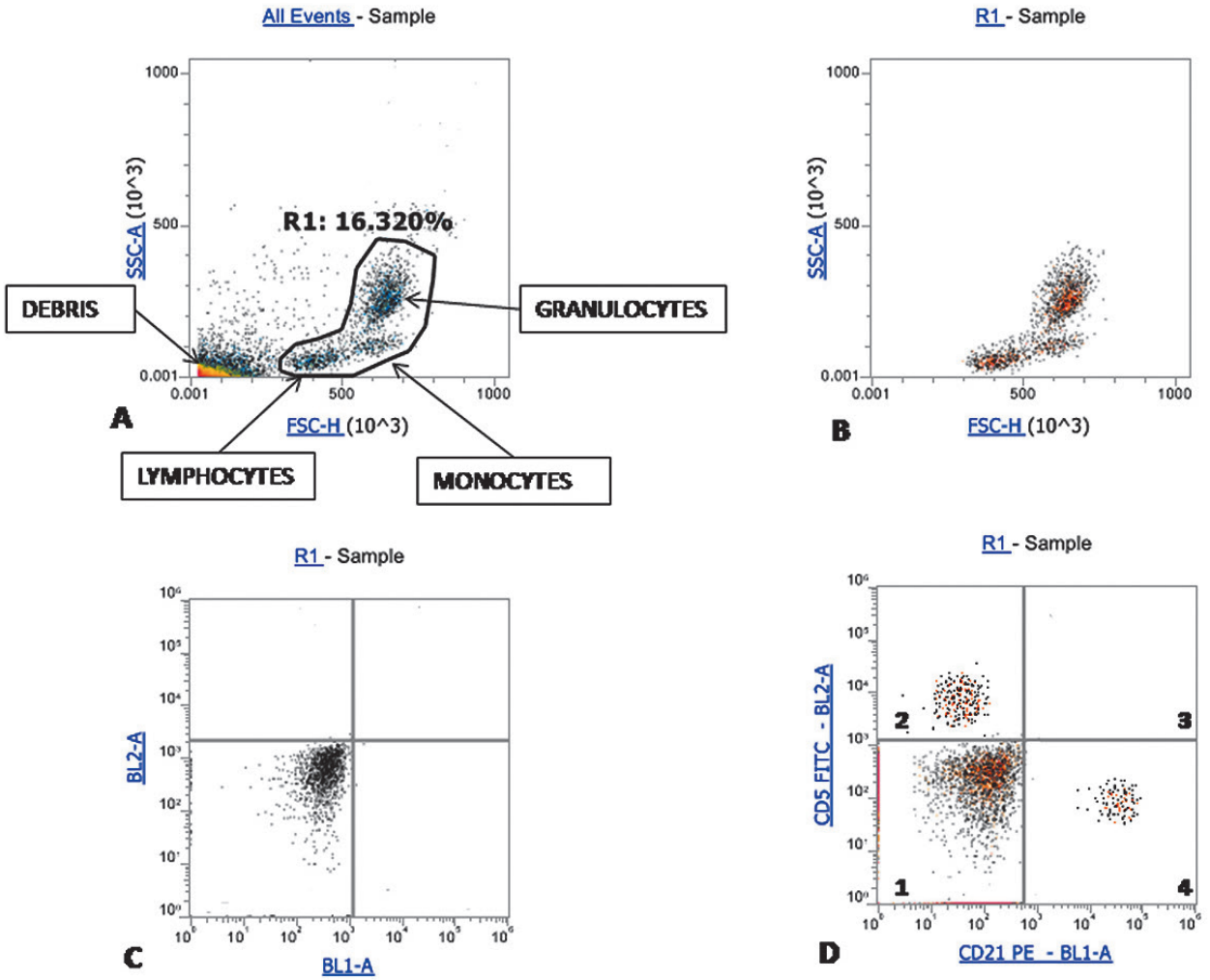

Figure 1: Normal peripheral blood sample dot plot routinely analyzed with an NxT Attune Flow Cytometer. Forward scatter (FSC) indicates cell size while side scatter (SSC) indicates cell granularity or complexity (A)

A - Gate of analysis designed to exclude debris from evaluation. Debris are presented as the less complex and smallest events (out of the analysis gate R1) followed by lymphocytes, monocytes and granulocytes.
B - The gate of analysis R1 activated in another dot plot in order to have a clear view of the cells of interest
C - FL1 and FL2 without application of antibodies
D - Two antibodies are applied CD5 (T-Cell Lymphocytes) and CD21 (Mature B-Cell Lymphocytes). Granulocytes are negative to both antibodies (quadrant 1), T-Cells positive to CD5 (quadrant 2), no events positive to CD5 and CD21 are present (quadrant 3), and B-Cells positive to CD21

Sample preparation for flow cytometric analysis

Since cells morphology can alter during conservation or transportation, sample analysis with flow cytometer should take place as soon as possible, but a period of time up to 24 hours from collection is considered suitable for the sample to maintain a good quality. Samples of Lymph nodes (LNs) and MCTs are collected using fine needle aspiration biopsy (FNAB) technique and placed in RPMI 1640 tubes (24, 25). For lymphoma usually seven to eight mass aspirations should be enough to collect an adequate number of cells for the analysis, while for MCTs more mass aspirations should be applied since mast cells are fragile compared to other white blood cells and furthermore degranulation can occur spontaneously in these cells. Peripheral blood and bone marrow samples are placed in ethylenediamine-tetra acetic acid solution (EDTA) tubes in order to prevent cell clotting.

Whenever possible it is strongly recommended to make cytological smears at the same time of sample collection. Before performing flow cytometric analysis it is necessary to quantify

the total number of cells/µl. For this purpose cell count analyzers (26, 27) or a flow cytometer can be used (28). A total number of 10^6 cells/ tube are considered adequate for the analysis of lymphomas and leukemia's, while for MCTs a minimum of 30 cells/µl is required to consider the sample suitable for the analysis (29).

Cells can be prepared for both staining methods, superficial and intracellular, depending on the specific analysis (30). In order to create a well-designed gate, to exclude debris from the analysis and to evaluate the samples quality, propidium iodide (PI) which binds the DNA of the fragmented cells can be used (31).

For the superficial labeling, after cell count has been performed, an aliquot of each sample is added to the FC tubes containing the specific monoclonal antibodies. Cells with the antibodies incubate at 4° C in a dark environment for 20 - 30 minutes. After incubation time, a red blood cells (RBC) lysis step is performed, using ammonium chloride lysis buffer since, RBC can interfere with the gate of analysis when white blood cells are analyzed. RBC lysis is not performed or it is modified in cases when erythroid origin leukemia is suspected. Lysis step lasts from five minutes in cases of MCTs to fifteen or more depending on the concentration of RBC in the sample. In some cases when analyzing peripheral blood or bone marrow, lysis can be done prior to the incubation, and RPMI medium can be added in order to stabilize the sample depending on the nature of the analysis. After lysis, samples are centrifuged usually at 1200 RMP for 5 minutes and then the supernatant is discarded while the pellet is resuspended with phosphate buffered saline (PBS). Lysis and PBS solutions are commercially available and ready for use, but they could also be prepared by own laboratory.

On the other hand, if intracellular staining is applied different steps including permeabilization of the membrane and fixation of the cells are necessary. Usually intracellular staining is used to detect intracellular cluster of differentiation (CD) or intracellular markers such as Ki67 and DNA content (32, 24).

Antibodies

Antibodies used in veterinary medicine can be both conjugated and non-conjugated. When antibodies are non-conjugated, a fluorochrome should be added since the laser can excite the fluorchrome conjugated to the antibody and not the antibody itself. Most common fluorochromes used in veterinary medicine include fluorescein (FITC), allophycocyanin (APC), phycoerythrin (PE), PE tandem dyes (PE-Cy5) and Alexa Fluor (AF488 and AF647) (33). Prior to its use, an antibody should always be titrated in order to exclude false positive signals.

Usually, antibodies used in FC are chosen based on the target cells under investigation. When a lymphoma is suspected the target cells are lymphocytes. Based on this criteria, specific antibodies which detect certain CD (CD3, CD5, CD4, CD8, CD34, CD21, CD45, MHC-II) are routinely used for the superficial labeling, while CyCD79b and CyCD3 are used for intracellular antigen detection (34, 35). Often these antibodies are combined together and placed in the same tube in order to make possible a multiparametric analysis. Thus, CD5, CD21, CD34, and CD45 can be placed in the same tube to detect the nature of the lymphocytes under investigation, and after that CD3, CD4, CD8 can be used if a CD5 positive lymphoma is identified. Different lymphomas can express different immunophenotypes (Table 1).

When leukemia is suspected a set of antibodies are used as a first approach and later other antibodies can be added, depending of the first analysis results. Since in cases of leukemia any of the cell lineages can be interested, more antibodies are needed for the immunophenotype characterization. For this purpose antibodies such as CD11b, CD14, CD61, CD90, CD5, CD21, CD11b, CD34, CD45 and CD44 can be applied (36). Staging of the tumors is made based on the

Table 1: Most common immunophenotypes expressed in main types of dog lymphomas.

Lymphomas	Common immunophenotype detected
B-Cell Lymphoma	CD45-Positive, CD21-Positive, CD34 Negative CD5,CD3,CD4,CD8-Negative,
T-Cell Lymphoma	CD45-Positive, CD21-Negative, CD34 Negative CD5,CD3-Positive
T-Zone Lymphoma	CD45-Negative, CD21-Positive, CD5-Positive, CD34-Negative

Table 2: Surface and intracellular antibodies used in cases of lymphoma and leukemia in dogs

Antibodies	Main Target Cells
CD5	T – Cells
CD3	T – Cells
CD4	Helper T – Cells
CD8	Cytotoxic T – Cells
CD21	Mature B – Cells
CD45	All Leukocytes
CD34	Hematopoietic stem cells
CD11b	Common myeloid marker
CD14	Monocytes
CD61	Platelets, Leukocytes, Endothelial cells
CDcy79b	B – Cells
CDcy3	T – Cells

immunophenotype found in the primary mass in case of lymphoma, and in the peripheral blood or bone marrow in leukemia cases. Most common antibodies used in veterinary medicine for lymphoma and leukemia are described in Table 2. When a MCTs is suspected specific antibodies for the identification of mast cells are needed. The first antibodies used are Immunoglobulin E (IgE) and CD117 since they have a high specificity for mast cells, especially CD117. These antibodies can be combined with primary lymphoid markers (CD5, CD21) when staging of MCTs take place in lymph-nodes in order to exclude lymphocytes from the gate of analysis. Pan leukocyte and myeloid markers can be added since mast cells have a myeloid nature. A list of antibodies used in MCTs in dogs is found in Table 3.

Table 3: List of superficial antibodies used in dog MCT

Antibodies	Marked cells
IgE	Mast cells, Basophils, Eosinophils, Macrophages
CD117	Mast cells, Basophils, Common Myeloid Progenitors, Multipotent Progenitors, Hematopoietic Stem Cells
CD18	Pan Leukocyte Marker
CD11b	Common myeloid marker
CD44	Most Hematopoietic Cells
CD5	Mature T - Cells
CD21	Mature B – Cells

Practical applicability of flow cytometry

One of the advantages of flow cytometry is the possibility to perform multiparametric analysis, which gives the possibility to analyze a group of cell populations for different marker expression at the same time. The first application of flow cytometry on lymphomas, leukemias and MCTs is the immunophenotypic characterization. Immunophenotype of the tumor provides information regarding the cell composition and nature of each tumor. In some cases, such as in chronic lymphocytic leukemia, the immunophenotype can predict the survival time (29). After exploring immunophenotype characteristics of the population and confirming diagnosis, the staging of the tumor takes place. Minimal residual disease can be performed after a specific chemotherapeutical protocol or treatment to observe the remaining percentage of the malignant cells.

Staging of lymphoma is usually performed on liver, spleen, peripheral blood and bone marrow. Flow cytometric staging is based on the immunophenotype of the specific lymphoma. When a diffuse large B cell lymphoma (DLBCL) diagnosis is confirmed, staging will be based in finding large CD21 positive cells in all tissues. Staging can be based also in aberrancies of the cells such as the presence of CD34 positive cells. In other cases, such as T-Zone lymphomas, it is based on the unique lymphoma immunophenotype (CD5+, CD21+ Low, CD45-). (22). According to the Canine Lymphoma Network, the majority of clinicians reported the definition of immunophenotype as the main reason for requiring flow cytometry, followed by the lymphoma subtype definition, checking minimal residual disease and differentiating lymphoma from reactive conditions (37).

Same as for lymphomas, the staging of MCTs is based on the immunophenotypic characteristics. Absence of normal cell antigens or presence of aberrant markers can be used to detect mast cells in tissues. Staging of MCTs in lymph nodes, peripheral blood and bone marrow is quite challenging due to the fact that mast cells can be present in normal conditions or in a reactive lymph node situation. However, flow cytometry is able to easily identify and quantify mast cells in lymph nodes (25).

Challenges and pitfalls of Flow Cytometry

Despite all the advantages mentioned in the previous sections, the Flow Cytometry technique has various challenges that have to be addressed when a new experiment take place, especially if using the technique for the first time.

Mainly, these challenges are related to technical and experimental design issues. One of the main problems when performing a new experiment is the choice of the antibodies and reagents. Antibodies should be chosen very carefully and in fully accordance with the cell populations of the researcher interest. Further, antibodies should be stored in dark in 4° C (38). Parameters of the owned FC should be always taken in consideration when purchasing conjugated antibodies, since the color and the number of lasers determine which fluorochrome can be excited by one specific FC.

Collection and manipulation of samples can be a challenge for the researches. When cells are separated such as in peripheral blood, bone marrow, and lymph node samples, FC analysis can be performed quite easily comparing with samples from solid tissues which needs a certain treatment (chemical or not) to separate the cells.

Results provided from Flow Cytometry are considered very accurate, but in many cases there are discordances with other techniques such as immunohistochemistry (39). Many antibodies that works for FC does not work for IHC and vice versa. Thus, interpretation of FC results with other techniques results may be challenging.

As a conclusion, all procedures that helps performing the analysis in an adequate way such as: use of propidium iodide to exclude debris, designation of a gate of analysis, fluorescence minus one procedure, use of isotype controls, choose of the antibodies and reagents, and designation of an experiment, needs a continuous commitment from the users to better determine and apply all of the above.

Conclusion

This is a first overview which aims to highlight the usefulness of Flow Cytometry, its importance and accessibility of using this method in the Balkan area conditions as the new generation of Flow Cytometers are getting more affordable for the research facilities and private laboratories.

Based on the authors experience and literature review, the flow cytometry technique is a coherent and useful method which can improve the diagnostic and research work. Flow Cytometry can identify and stage lymphomas, leukemia's and mast cell tumors. Multiparametric analysis, large number of events analyzed in a short period of time and the fast turn-around time to provide results, makes flow cytometry particularly appealing for the routine diagnosis of these malignancies. The technique is compatible with other methods, but results provided by FC must be interpreted along with data gained by histopathology, immunohistochemistry and cytology, in order to have a large and valuable quantity of information.

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UPORABNOST PRETOČNE CITOMETRIJE PRI PREPOZNAVANJU IN DOLOČANJU STADIJA LIMFOMA, LEVKEMIJE IN TUMORJEV MASTOCITOV PRI PSIH – PREGLED

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Izvleček: Limfomi, levkemije in tumorji mastocitov so najpomembnejše skupine neoplazem, ki prizadenejo pse. Diagnostika, določanje stopenj tumorja in tipa celic v določenem tumorju predstavljajo izziv za raziskovalce in klinike in igrajo ključno vlogo pri učinkovitosti zdravljenja in postavljanju prognoze. Za rutinsko diagnosticiranje in določanje stopenj teh tumorjev se uporablja veliko različnih temeljnih metod, kot so citologija, histopatologija, imunohistokemija in citokemija. V zadnjih letih je pretočna citometrija vse bolj uporabljana metoda v veterinarski medicini, saj je mogoče v kratkem času in z visoko natančnostjo analizirati veliko število zdravstvenih stanj. Ena izmed najpomembnejših prednosti te tehnike je multiparametrična analiza, s katero lahko v populaciji celic istočasno analiziramo različne površinske označevalce. Določanje površinskih označevalcev in stopenj populacij tumorskih celic s pretočno citometrijo lahko pripomore k potrditvi diagnoze in postavitvi ustrezne prognoze bolezni. Številni rezultati pretočne citometrije so imeli pomemben prognostični pomen zlasti pri neoplastičnih obolenjih. Vendar je rezultate pretočne citometrije potrebno združiti in razlagati v skladu s podatki, pridobljenimi s histopatologijo, imunohistokemijo in citologijo.

Ključne besede: pretočna citometrija; protitelesa; diagnoza; limfom; levkemija