Applications of Flow Cytometry in Veterinary Medicine

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Overview

1. Instrumentation
   A. Essential components
   B. Basic principles of light scatter and fluorescence

2. Sample preparation/processing

3. Application of flow cytometry
   A. Detection of surface antibody
   B. Lineage determination of malignant neoplasms
   C. Define an antibody panel
   D. Identifying clonality (PARR test)

4. Case examples
Flow cytometers

• “flow cyto – meter” = measurement of cells in a fluid stream
  – Measure multiple characteristics of cells by light scatter and fluorescence using lasers
  – Quantify leukocytes and differentiate cell types
  – Fluorescent dye labeled antibodies to cluster of differentiation antigens identify subsets of cells
    • i.e. CD4 or CD8 lymphocytes
      – immunodeficiency syndromes
      – lineage of lymphoma and leukemia
  • DNA content, apoptosis, viability, metabolism, proliferation
First Flow Cytometer, an “old tool”

4. Electronics

Basic Components
1. Laser – excitation optics
2. Fluidics
3. Collection Optics
4. Electronics

Analytical Flow Cytometer
FACSCalibur  BD
Components of a bench top analytical flow cytometer

- Fluidics
- Laser
- Collection Optics/Electronics
- Sample tube
- Flow cell
Light Amplification by Stimulated Emission of Radiation

UV Laser

Wavelength (nm)

Argon Laser

FITC GFP

PE

Red Diode Laser

APC

K-State
Fluorescence colors

- First fluorochromes or fluorophores
  - Fluorescein (FITC)
  - Seaweeds/cyanobacteria
    - Phycobiliproteins (PE)
    - Allophycocyanin (APC)
  - Jelly fish - Green fluorescent protein (GFP)
  - Propidium iodide (PI)
Fluidics

- Pressure lines and sheath fluid introduce sample stream into laser beam
- Sample pressure is higher than sheath fluid and introduces cells in single file

Coaxial flow
Basic light scatter properties of cells

$90^\circ$ Light Scatter (Side Scatter) $\propto$ complexity

Granularity

Forward Scatter $\propto$ Cell Size

Laser Beam
Differential light scatter properties of cells define individual cells
Hematology Analyzers
Advia 2120i use laser light scatter to differentiate cell populations

<table>
<thead>
<tr>
<th>Size</th>
<th>Complexity</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBCS/platelets</td>
<td>Dog: WBC 9.3x10^9/1</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td></td>
</tr>
<tr>
<td>Monocytes</td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td></td>
</tr>
<tr>
<td>Eosinophils</td>
<td></td>
</tr>
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</table>
Collection Optics

- Collection Optics
  - Mirrors, Filters, Detectors
  - Collect and filter wavelengths of light that come from the particle-laser beam interaction
    - Light scatter
    - Fluorescence
Basic principles of fluorescence

<table>
<thead>
<tr>
<th>Argon laser $\lambda = 488$ nm</th>
<th>emitted fluorescent light energy $\lambda = 530$ nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fluorescent Intensity $\propto$ binding sites</td>
</tr>
</tbody>
</table>

Fluorescence

Laser excites fluorescently labeled antibodies to CD molecules
Large granular lymphocytosis

Lymphocytosis = 13,000/μL

Does the dog have Ehrlichiosis or lymphocytic leukemia?

When should immunophenotyping be ordered?

• 2006 Consensus recommendations to use FC IP to screen for malignancy in people:
  – Bicytopenia and pancytopenia
  – Elevated leukocyte concentration
  – Presence of atypical cells or blasts in blood, bone marrow, or body fluids
  – Plasmacytosis or monoclonal gammopathy
  – Organomegaly and tissue masses
Key Role in Diagnosis and Classification

- Mature lymphoid neoplasms
- Plasma cell neoplasms
- Blastic malignancies
- Maturing myeloid and monocytic malignancies
- Monitoring treatment of a previously diagnosed hematolymphoid neoplasia
- Detection of minimal residual disease
  - 0.1 to 0.01% abnormal cells
Immunophenotyping in Veterinary Medicine

- Immunophenotyping using monoclonal antibodies to cluster differentiation (CD) molecules better defines the lineage of hemic neoplasia than morphologic assessment
  - B lymphomas respond more readily to therapy
  - Leukemias can be differentiated into
    - Myeloid vs. Lymphocytic
    - Acute leukemias identified by CD34 expression
    - Chronic lymphocytic leukemia in dogs is principally a CD8+ cell lineage (CD4+ in cats)
Cell lineage differentiation defined by Cluster of Differentiation molecules

**Myeloid**
- CD11b
- myeloperoxidase

**Monocytic**
- CD14

**Dendritic**
- CD11c, CD1a
- CD4, CD18

**Lymphocytic**

- Pro-B
- Pre-B
- Immature B cell
- Mature B
- Plasma cell

- CD45R
- MHCII
- CD79a,b
- sIgM
- CD21

- CD4
- CD8

- CD4, CD8
- Pro-thymocytes
- Cortical thymocyte
- Medullary thymocyte
- Mature T cells
### Selecting an Antibody Panel

<table>
<thead>
<tr>
<th>CD molecule</th>
<th>T cell</th>
<th>B cell</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td></td>
<td></td>
<td>Pan leukocyte</td>
</tr>
<tr>
<td>CD34</td>
<td></td>
<td></td>
<td>Stem cell</td>
</tr>
<tr>
<td>CD3 or CD5</td>
<td>T cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>T helper</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8</td>
<td>T cytotoxic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD79a (cytoplasmic)</td>
<td></td>
<td>Pro B cell to Plasma cell</td>
<td></td>
</tr>
<tr>
<td>sIgM, CD22</td>
<td></td>
<td>Immat./Mat. B</td>
<td></td>
</tr>
<tr>
<td>CD21</td>
<td></td>
<td>Mature B</td>
<td></td>
</tr>
<tr>
<td>CD14</td>
<td></td>
<td></td>
<td>Monocytes</td>
</tr>
<tr>
<td>CD11b</td>
<td></td>
<td></td>
<td>Granulocytes</td>
</tr>
<tr>
<td>CD11c/CD1a</td>
<td></td>
<td></td>
<td>Dendritic cells</td>
</tr>
</tbody>
</table>
Sample preparation and processing

- Blood or bone marrow in EDTA anticoagulated collection tubes
- Tissue samples (lymph nodes)
  - Needle biopsy of multiple areas
  - Best to have 500,000 to 1 million cells/mL
  - Add sample to a tube of 1 – 2 mL of saline
  - Send overnight carrier on cool packs
Scatter Plot of blood leukocytes

Forward Scatter $\propto$ Size

Side Scatter $\propto$ Complexity

Granulocytes

Plts
Dead cells

Monocytes

Lymphocytes
Leukemia

13 yr Spaniel
WBC = 120,000/µL

Acute Leukemia (CD34+)
Possible Myelomonocytic
Guidelines for lymphoid malignancy

• In blood, lymphocyte concentration above reference range for lab and one of the following
  • 80% of lymphocytes with a single phenotype or
  • 60% of lymphocytes with a single phenotype and a positive clonality assay (PARR) or
  • Presence of lymphocytes with an aberrant phenotype for peripheral blood

• In tissue
  – Expansion of homogenous population of single phenotype
  – Presence of aberrant phenotype
Immunophenotyping of Fluids

- Cerebral spinal fluid
- Dog with ataxia
- Epidural lesion at T4
- Total nucleated cell count = 3,175/µL
- Protein = 145 mg/dL
- Cytology
  - Pleocytocysis
  - Monocytic or lymphocytic?
Diagnosis of CSF case

• Immunophenotyping panel
  – CD45 (pan leukocyte) 99% cells
  – CD3+ (T cell marker) 87% cells
  – CD21 (mature B cell) 3% cells
  – CD14 (monocyte) 18% cells

• Necropsy
  – Multicentric lymphoma
  – Lymph nodes and Liver
  – Invasion of spinal nerves
Adult Dog with mediastinal mass

Cells from needle aspirate labeled with fluorescent antibodies to CD4 and CD8
- Thymic lymphoma?
- B cell lymphoma?
- Thymoma? 😞

Is this lesion polyclonal or monoclonal? What would you expect with PARR?
See Lana et al., 2006 JVIM and Lara-Garcia et al., 2008 VCP.
Antibody specificity

- Dot plot analysis
  - Complexity vs. size
    - Place gates on cells based on size
    - Green = Small cells
    - Pink = Large cells
  - Size vs. Fluorescence
    - Cells stained for CD markers are detected to the right of the vertical line
Lymphoma cases

- 5 yr old Labrador
- Large lymphoblastic cells
  - CD3 negative
  - CD21+ IgM+

B cell lineage
Case 2

9 year old mix breed dog
- Large & small cells
  - CD3+ CD4+
- Few small cells (resident)
  - CD8+
  - CD21+CD79a+

T cell lineage

CD3
CD4
CD8
CD21
CD79a

Fluorescence
Case 3
7 year old Pointer

- Large cells
  - CD34+ CD21+ IgM+ CD79a wk+
- Small cells
  - CD3+
- Significance of CD34?
- Which cells are neoplastic?
Significance of CD34 expression in human lymphoma

- Schmidt et al., *Aberrant Antigen Expression Detected by Multiparameter Three Color Flow Cytometry in Intermediate and High Grade B-Cell Lymphomas*. 1999. Leukemia and Lymphoma

15% of the B cell lymphomas were CD34+
Immunophenotypic markers of prognosis in canine lymphoproliferative disorders

- Study at CSU of 96 dogs with lymphocytosis including stage V lymphoma. Williams et. al. 2008. JVIM
  - CD34+ phenotype in the blood had shorter survival time (average of 16 days) compared to lymphocytosis that expressed mature B and T cell antigens (300 – 500 days)
  - CD21+ lymphocytosis composed of large cells had shorter survival times compared to small cell CD21+ lymphocytosis
- Rao, et. al., JVIM. 2011
  - Lack of MHC II expression in B cell lymphomas had a poor outcome (Rao, et al., JVIM. 2011.)
  - T cell lymphomas not expressing CD5 had a better prognosis
Case 4: 2-year old male Golden retriever with peripheral lymphadenopathy

**Laboratory Data Abnormalities**

Hypercalcemia [19.4 mg/dL]  Ref. Range [9.7-12.1 mg/dL]
Thrombocytopenia 36,000/µL  Ref. Range [164,000-10,000/µL]
Lymphopenia 800/µL  Ref. Range [1,500-5,000/µL]
Aspirate of prescapular lymph node

90% of cells are immature lymphocytes

Neutrophil

25μm
Bone Marrow Aspirate

Immature lymphocytes replace normal bone marrow
High power image of Bone Marrow

- Segmented neutrophil
- Band neutrophil
- Hand Mirror shape to immature lymphocytes
Diagnosis = Stage V lymphoma
Additional Tests: Immunophenotyping using flow cytometry to determine cell lineage

Majority of lymphocytes in lymph node aspirate express T cell antigens (CD5, CD3, CD4)
Cell lineage = ?

Hypercalcemia common with T Cell Lymphomas
Lymphoma vs reactive lymph node?

• Lymphoma = neoplasia of lymphocytes
  – Clonal expansion of T or B cells
    • Single receptor specificity
    • Immature morphology

• Reactive lymph node
  – Antigenic stimulation causes expansion of multiple clones of T and B cells (polyclonal)
    • Multiple receptor specificities
    • Mature morphology and presence of plasma cells
Reactive lymph node

- Mast cell
- Russell bodies
- Immature lymphocyte
- Mature lymphocytes
- Plasma cells
How do you distinguish reactive lymph node enlargement from lymphoma?

- Grossly bilateral lymphadenopathy suggests neoplasia
PCR for antigen receptor (PARR) rearrangements to identify clonality

PCR primers amplify products of various sizes due to varying inserts of nucleotide additions

TCR, BCR (Ig) genes

Single band indicates monoclonal population of T cells

PCR primers

Primers amplify antigen binding site

Avery, CSU
Light chain or α

Germline DNA → V1-40, J1-6, C → DJ rearranged → VJ or V-DJ → mRNA

Recombination

Heavy chain or β

Germline DNA introns → V1-51, D1-27, J1-5, CH1, CH2, CH3 → DJ rearranged → V-DJ → mRNA

BCR or Antibody protein

CDR3 encodes for Ag binding site

K-STATE
PCR for antigen rearrangement

Primers
Amplify
B cell clone

Primers
amplify
T cell clone

Cμ  Ig  TCR

A  B  C

Polyclonal

Dr. Avery
CSU
Important information on the PARR test

1. Sensitivity & Specificity: 75% & 92%
2. Detects 1 neoplastic lymphocytes in 100 heterogeneous nonneoplastic cells
3. Detects neoplastic lymphocytes in PB 2.5 times more than microscopic evaluation
4. Not prognostic for disease-free interval or duration of survival
5. Confirmed B-cell lymphosarcoma in aqueous humor sample
6. **False negatives** – primers not specific, somatic recombination, NK cells
7. **False positive** - pseudoclonality (amount and quality of DNA)

• Burnett et al., 2003 Vet. Path.
• Lana et al., 2006. JVIM
• Pate et al., 2011. JAVMA
• Werner et al., 2005 Vet. Path.
Case 5 Mixture of B and T cells
Large B lymphocytes are clonal

T cell Rich
B cell Lymphoma
Case 6
7 yr Labrador cross

- **Large & small cells**
  - CD3+, CD34+

- **Small cells**
  - CD4+, CD8+ bright

- **Large cells**
  - CD21+/CD3+, CD79a+

B cell lineage

CD3

CD4

CD8

CD21

CD34

CD79a

Fluorescence
Case 7: Aberrant T cell and monocyte antigen expression in a B cell clone

PARR indicates B cell clonality
Reports of aberrant CD expression and gene rearrangements

• Gelain et al., 2008. Aberrant phenotypes and quantitative antigen expression in different subtypes of canine lymphoma by flow cytometry.
  – B – decreased CD79 and expression of CD34
  – T – decreased CD45 expression
  – CD3 or CD5 expression without CD4 or CD8
  – CD79 and not CD21 for B cells

• Wilkerson et al., 2005. Lineage differentiation of canine lymphoma/leukemias and aberrant expression of CD molecules.
  – CD8, CD14, and CD21

Conclusions

1. Flow cytometry Immunophenotyping useful tool in diagnosis/prognosis of canine lymphoproliferative and hematopoietic neoplasias
   • Providing the sample can be dispersed in suspension
   • Correlates with cytomorphologic features

2. Broad immunophenotyping panels and multi-color analysis improves diagnostic capabilities.

3. Lineage infidelity is common at early stages of hematopoietic differentiation. Aberrant expression of CD molecules occurs in canine lymphomas/leukemias and could be used to screen for neoplastic disorders, minimal residual disease or monitor relapse.

4. PARR done when cytology, histopathology, and immunophenotyping is ambiguous.
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