Flow cytometry in acute leukemia

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Abstract Immunophenotyping of acute leukemia is one of the most important clinical application of Flow cytometry. The aim of this work is to review recent advances in flow cytometry methods, quality control, troubleshooting and its prevention and data analysis of acute leukemia. Multiparameter flow cytometry is a useful adjunct to morphology and cytochemistry and it is an invaluable tool in the diagnosis of acute leukemia.

Keywords Flow cytometry · Immunophenotyping · Acute leukemia · Leukemia

Introduction

Flow cytometry is the methodology used to detect cell surface antigens using monoclonal antibodies conjugated with different fluorochromes. The value of characterization of leukemic cell is to establish the lineage (such as myeloid, T lymphoid or B lymphoid). It would also be used to establish the dual lineage in biphenotypic leukemia, unusual coexpression of antigens or aberrant profile and demonstrate clonality.

Immunophenotyping can be done using various methods such as immunofluorescence method, alkaline phosphatase anti alkaline phosphatase (APAAP) technique or flow cytometry.

There have been several recent advances in flow-cytometric technique such as the use of the multicolor (5/6/9/10 color) flow cytometer [1], gating strategies by using CD 45, new techniques using lyse and wash method, advanced software and expanded range of antibodies, analysis by looking pattern of expression and percentage of positivity. Thus making it the most preferred Immunophenotyping mode.

The current review deals with issues related to selection of antibody panel, methods, gating, data interpretation, reporting, standardization, troubleshooting and its prevention pertaining to flow cytometry.

Selection of antibody panel

It is important to have set group of monoclonal antibodies selected specifically to use for different diseases, called as panel. The use of wrong panel, or one which is too small or restricted, often fails to define phenotype sufficiently

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or may miss an important differential diagnosis. In UK, the general hematology task force of the BCSH published recommendations for immunophenotyping of acute leukemia in 1994. The European Group for the Immunological Characterization of Leukemias (EGIL) published their proposals for the immunological classification in 1995. In practice, laboratories select their leukemia panel according to their workload cost of monoclonal antibodies. The panel of antibodies should include B and T lymphoid and myeloid markers [2] as given table 1.

Methods

Preparation of sample for flow cytometry

Two ml of blood or bone marrow is required in dipotassium ethylene diamine tetra acetic and K2EDTA or heparin. Sample should be properly shaken to prevent clots. Each sample should be shaken 5–10 times upside down before processing. Slide should be prepared and stained with Jeiner-Giemsa for each flow cytometric specimen. Viability of cells can also be done if required by using trypan blue stain. Following this, further processing of cells can be done using whole blood lyse-no-wash method or whole blood lyse-and-wash method.

Lyse-no-wash methods minimizes the loss of cells in the samples. Lyse causes lysis of red blood cells. Usual number of cells recommended for immunostaining is 10^6 cells for each test tube antibody cocktail [3].

Pattern recognition and gating strategies

For a particular monoclonal antibody, intensity of antigen expression as well as percentage of cells that are positive are accounted for. The traditional side scatter and forward scatter SSC/FSC gating is still widely used. However, gating by CD45/SSC is more accurate [4]. Other options are

sequential and back gating. In the case of AML M0 and M1, blasts have FSC and SSC and demonstrate moderate intense expression of CD45; in cases of myeloid leukemia with granulocytic differentiation, CD45 and SSC shows dispersed blasts population merging with maturing granulocytic elements. In cases of monocytic leukemias, blasts show overlap with the region where normal monocytes are found [5].

In acute lymphoblastic leukemia lymphoblasts are CD45 low or negative with low SSC [6].

Data interpretation

It is customary to report the percentage of blasts expressing each antigen tested and to consider any markers present in more than 20% of blasts as positive. Though this cut off point of 20% is arbitrary. However, another way of analysis is by intensity of antigen expression i.e. antigen binding capacity of a given fluorochrome antibody conjugate. The first log decalog (between 10^0 and 10^1) represents negative fluorescence and signals falling in the second, third and fourth decades represents weak (+), moderate (++), and strong(+++) fluorescence intensity, respectively. Sometimes, because of heterogenous staining intensity, malignant cell clusters may appear bimodal [3]. It becomes problematic to report the fluorescence intensity, then the brighter half of the bimodal distribution provides the more clinically relevant information and the fluorescence intensity of the brighter half can be reported by adding bimodal as a modifier.. One should take care of the parameters such as the threshold and voltage.

Reporting

Reporting should be a composite one including morphology, cytochemistry and immunophenotype. Report should include date, time of receipt at laboratory, quality of sample

Table 1 Panel of monoclonal antibodies for the diagnosis of acute leukemia

	ALL		AML
	B lineage	T lineage	CD13, CD33, CD117, Anti MPO
First line			TdT, HLADR, CD34
	CD19, CD22, CD79a, CD10	CD7, CD2, CD3	
Second line	Cymu, SmIg	CD1a, CD5, CD4, CD8, anti TCR	CD41, CD42, CD61, anti glycophorin A

ALL, acute lymphoblastic leukemia; AML, Acute myeloid leukemia; CD, Clusters of differentiation; Cy, Cytoplasmic; SmIg, Smigsurface, immunoglobulin; TdT, Terminal Deoxynucleatidyl transferase; TCR, T Cell receptor; MPO, Myeloperoxidase; Optional markers: CD14, antilysozyme, CD36 CD10 and cymu are not essential for the diagnosis of B lineage ALL, but they are important in paediatric cases to identify common ALL, pro-B ALL and pre-B ALL.

and acquisition time, type of technique used, type of gating done.

Standardization and compensation

Standardization can be done by 7 color set up beads (commercially available, as multi color BD FACS Canto) flow cytometer. Expired beads should not be used. Set up beads are prepared by bead diluent containing sodium azide as preservative. Kit should be kept at 2–8°C. Beads are run on FACS Canto clinical software, During set up, these beads are used by software to adjust voltages that ensures that fluorescence brightness is correct for stained cells in each detector. Along with that, it corrects spectral overlap as compare to the newly established spectral overlap volumes, which is used by the software to ensure that stained cells are correctly compensated. Rainbow beads can also be used.

Trouble shooting and prevention

Commonest trouble shooting is connection of cytometer to the software. It is better to check connections of all wires, switch off and again restart. It is always better to acquire rinse and then distill water during end of the day or start of the work. Sometimes there is no population seen on FSC/SSC. Then also one should stop acquiring and run cleaning solution for 5–7 min. It is always better to save all the data in D drive and keep back up in two sets of compact disc. Sometimes fan in the cytometer also produces noise. Compressed Processing Unit (CPU) may not start at time, so it is better to delete temporary files on weekly basis. Hard disc should be checked every 1–1.5 year to know the space in disc. Full hard disc can be changed depending upon the work load in the laboratory. Room in the lab with a flow cytometer should not be a eating place otherwise rats can cause damage to wires, which are very costly. While acquiring sample, sometimes when you don't observe population of cells, a new folder and experiment should be made to run sample.

Acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) constitutes a heterogeneous group of malignancies that share a common lymphoid origin. Morphologic and cytochemical analysis is necessary for the diagnostic evaluation, but immunophenotyping is always essential for lineage assignment and to differentiate ALL from certain type of acute myeloid leukemia (AML)

[6]. The lymphoblast in B lineage ALL are terminal deoxy nucleotidyl transferase positive, HLA-DR positive, and almost always positive for CD10 and cytoplasmic CD79a [7]. Cases of ALL expressing myeloid related antigens such as CD33, CD13 or CD117 are extremely rare and by definition always negative for myeloperoxidase [5]. In childhood ALL, the B precursor phenotype has been associated with relatively favorable prognosis, with the exception of CD10 negative ALL specially in infants. T cell phenotype is present in 25% adult and 15% childhood ALL. CD7 is the single most sensitive surface marker for T lineage differentiation [5] but since it can expressed on myeloid leukemia and therefore, can not define T ALL. Most specific marker for T ALL is CD3. T ALLs are TdT positive and variably express CD1a, CD2, CD3, CD4, CD5, CD7 and CD8, CD10 may be positive, CD79a positivity has been observed in some cases [7].

T lymphoblast can demonstrate T cell receptor gene (TCR) but it is not lineage specific. The most reliable marker for T ALL is cytoplasmic CD3, but care must be taken not to overinterpret dim cytoplasmic staining [5].

Acute myeloid leukemia

Immunophenotypic analysis has a central role in distinguishing between minimally differentiated acute myeloid leukemia and acute lymphoblastic leukemia and in the recognition of acute megakaryoblastic leukemia. The ability of immunophenotyping to identify myeloid differentiation approaches 98% [5]. AML prominent blasts population expressing myeloid antigen, results of other markers such as HLA-DR, CD14, CD16, help in subclassifying AML. Blast population demonstrate variables distribution of CD34, CD13 or CD33, but it does not add much to the subclassification of AML. The diagnostic importance of HLA-DR lies in its lack of impression seen in AML-M3. While blasts in non M3 AML express moderate to bright levels of HLA-DR [3]. Dot plot of AML-M3 is shown in figure 1.

Absence of HLA-DR can occur in some cases of AML M1 [3]. In cases of monocytic differentiation CD13 and CD33 are most commonly expressed while CD34 and CD117 are generally absent [5], Monocytic leukemia express brighter CD33 than do other myeloid leukemia (except acute promyelocytic leukemia) [3] Monocytic leukemia typically express CD14 and CD64.

Megakaryoblasts demonstrate bright expression of CD61 and CD41, but one must exclude platelets adhering to leukemic myeloblasts. CD61 can be combined with CD42b which is strongly expressed on platelets but absent on megakaryoblasts [5].

PerCP-Cy5.5 peridinin chlorophyll Cy5.5
 PE: Phyco erythrin FSC: Forward scatter
 APC Allophycocynin SSC: Side scatter
 APC-Cy7- Allophycocynin Cy7

Fig. 1 Dot plot of AML – M3 shows population in forward scatter and side scatter, CD 45 gated blast and promyelomycytes and positivity of CD13 and CD33

Acute leukemia of ambiguous lineage

Bilineal acute leukemia is characterized by detection of a dual population of blasts with each population expressing markers of a distinct lineage such as myeloid and lymphoid or B and T, where both types are associated with poor outcome [8]. Biphenotypic acute leukemia are characterized by blasts which coexpress myeloid and T or B lineage specific antigens or B and T antigens. The EGIL suggested the use of scoring system based on different combination of antigen [9]. EGIL criteria is mainly designed to recognize cases of antigen coexpressoin and did not explain cases with dual leukemic population and distinguish them from those with shared antigens.

Conclusion

Majority of laboratories perform a panel adopted to their own practice. There is a need for a consensus in the selection of leukemia panels. Most important consideration is

right interpretation of the results and ensuring that reports of those results are given in a meaningful manner to the clinician.

The participation in quality assurance scheme to monitor the performance of laboratory and the people involved in the diagnosis of samples sent for immunophenotyping is another essential part of good laboratory practice.

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