technical note

How we assess adequacy of fine-needle aspiration materials intended for flow cytometric analysis

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Many articles have been published on the subject of FNA, highlighting the usefulness of flow cytometry in the diagnosis and classification of lymphomas. But occasionally, flow cytometric evaluation fails to detect an abnormal population in a FNA specimen involved by lymphoid neoplasm. Sampling errors (poor viability, peripheral blood contamination and hypocellular specimens) are the major reasons of this failure. In our laboratory we use a simple, fast and cost-effective approach to assess adequacy of FNA materials and in this paper, we describe this procedure with giving some examples of interpretations of our results.

Fine-needle aspiration (FNA) is a simple and painless procedure and FNA materials are very useful tools for the diagnosis classification and staging of newly diagnosed and recurrent lymphomas. Many articles have been published on the subject of FNA, highlighting the usefulness of flow cytometry in the diagnosis and classification of lymphomas.[1-5] Flow cytometry is suitable for liquid specimens such as blood, bone marrow and serous effusions (for example, peritoneal, pleura) which is not the case for the solid tumors such as lymph nodes, spleen, liver, and cutaneous nodules, which is why aspirates must be suspended in various solutions to analyze them by flow cytometry.

Occasionally, flow cytometric evaluation fails to detect an abnormal population in a FNA specimen involved by a lymphoid neoplasm. Sampling errors (poor viability, peripheral blood contamination and hypocellular specimens) are the major reasons of this failure.[6] Meda et al analysed 290 aspirates from 275 patients. Flow cytometric analysis was impossible in 14 cases (4.8%) due to sampling problems (poor viability in 6 cases, peripheral blood contamination in 5 cases and insufficient quantity in 3 cases).[7] According to Young et al, five FNAs out of 107 (4.7%) were deemed inadequate for diagnosis because of insufficient cellularity and were excluded from the study.[8] Zeppa et al studied 307 FNAs in which 15 cases (4.9%) were inadequate for flow cytometric analysis.[2]

In our laboratory we use a simple, fast and cost-effective approach to assess the adequacy of FNA materials and we describe this procedure, giving some examples of interpretations of our results.

Sampling technique

The FNA of subcutaneous lymph nodes and tumors are performed in the outpatient setting according to described methods.[9,10] Using a 30-gauge hypodermic needle with a 1mL syringe attached, we fixate the lymph node between two fingers of the free hand, insert the needle into the node, and apply forceful suction to aspirate a small amount of material from several locations, changing the angle of the needle slightly. The needle is removed if blood or cellular material is observed within the hub. Suction is maintained while the needle is withdrawn into the subcutis. Fine needle aspirates are diluted in a tube containing K3EDTA and 2 mL of phosphate-buffered saline (PBS) or RPMI medium. The cell suspension is then strained through a 35µm nylon mesh filter to remove large debris and clumps of cells.

In our experience, the cellular material obtained by

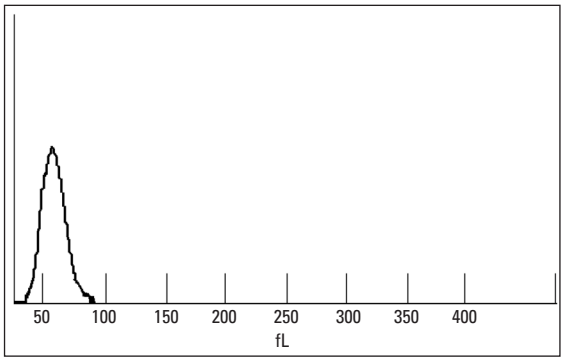

Figure 1. FNA sample of a patient with a mantel zone lymphoma: notice that there are no apoptotic bodies and cell sizes are included between 35 and 90 fL.

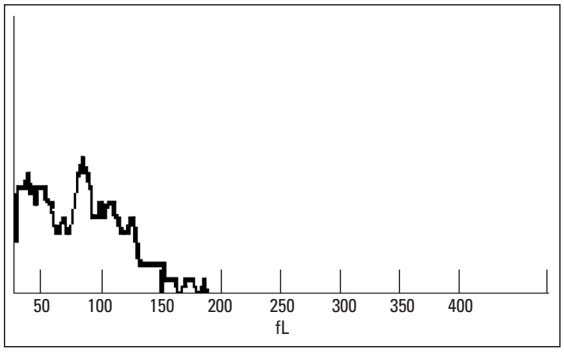

Figure 2. FNA sample of a patient with a diffuse large B-cell lymphomas: notice that there is an admixture of apoptotic bodies (less than 30 fL) small lymphoid cells (35 to 90 fL) and large lymphoid cells (more than 90 fl).

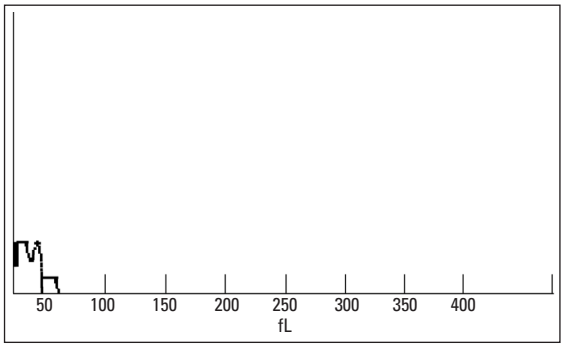

Figure 3. WBC histogram demonstrating only apoptotic bodies: notice that the majority of cells are smaller then 50 fL and that the peak does not start at the base line.

this method, contains more than one million viable lymphoid cells with few apoptotic bodies. In comparison to the so-called Zajicek or pipette technique which uses a needle alone without a syringe to gather more necrotic and apoptotic bodies.[11,12]

Quality control of the sample

An automated cell count of the sample is performed using an impedance cell counter (Coulter ACT Diff). This instrument demonstrates a three part predifferentiation leukocyte histogram, which is very helpful in assessing cellularity, blood contamination, lymphoid cells size and viability.

Cellularity

The specimen requirements of flow cytometry may vary somewhat from laboratory to laboratory. With highly cellular specimens, the common practice is to stain 1 million cells per tube, with data collected for 10 000 to 30 000 events. However, specimens with lower cellularity still may be amenable to analysis. At a minimum, approximately 50 000 cells per tube are needed to analyze 10 000 events. If a specimen contains a pure lymphoma population, even fewer cells may suffice for diagnosis.[13] In our laboratory, the policy is that the sample must contain at least 1000 lymphoid cells /μL. If 1000 lymphoid cells /μL are not available, another aspiration is immediately applied and rinsed into the sample.

Peripheral blood contamination

The majority of our samples contain an average of 10^4 to 2×10^4 erythrocytes/μL but it is predictable that the samples will enclose some red blood cells as we are applying forceful suction to aspirate a small amount of material. However the "lyse and wash" procedure will get rid of these red cells and the multi-parametric gating strategies will allow us to exclude the reactive lymphoid cells from the analysis. In our laboratory we prefer to evaluate blood contamination by calculating the granulocyte/lymphoid cell ratio, which must be lower than 0.2, because in samples with more lymphoid cells do not cause any problem during the cytometric analysis even if they are mixed with reactive or peripheral cells.

Cell size

Gong et al tried to evaluate the cell size of the neoplastic population by comparing them with the T-cell population on forward scatter histogram (FSC-H).[14] But many authors agree with the fact that large neoplastic cells are often disrupted during FNA and processing for flow cytometry.[7,13]

In one series of FNA studies for the primary diagnosis of lymphadenopathy, 27% of the cases that ultimately were shown to be large B-cell lymphomas initially were found to be negative for malignancy by flow cytometry.[15] In such cases, the examination of forward-scatter versus side-scatter plots demonstrated that large cells

usually are markedly reduced or absent, in comparison with their frequency on cytologic preparations. That is why in our unit we prefer to assess cell size before cytometric processing by examining the 3 part pre-differential leukocytes histogram.

Size-referenced white blood cell (WBC) histograms display the classification of leukocytes according to size following lysis, not the native cell size. The lytic agent lyses the cell and the cytoplasm collapses around the nucleus, producing differential shrinkage. The histogram subpopulations reflect the sorting of the cells by their relative size, which is primarily their nuclear size. The differentiation is as follows: lymphocytes (35-90 fL), mononuclear (90-160 fL) and granulocyte (160-450 fL).[16] **Figure 1** shows a WBC histogram in a patient with a mantel zone lymphoma, and in which the entire population falls in the lymphocyte area (between 35 and 90 fL). **Figure 2** shows a WBC histogram in a woman with a diffuse large B-cell lymphomas in which we found an admixture of small cells and large neoplastic cells measuring more than 90 fL and even reaching 200 fL.

Viability

The most classic methods of assessing cell viability are through the use of vital dyes. These are based on the loss of membrane integrity of dying cells; allowing access to dye that cannot penetrate a living cell.[17] The trypan blue technique is a noncytometric method in which the stain is mixed with the sample, and only the nonviable cells take up the stain. Nonviable blue-colored cells are then counted manually by light microscopy.[17-19]

By using the cytometric methods, viability may be assessed simultaneously with the analysis of surface marker expression using dyes such as propidium iodide (PI) or 7-amino-actinomycin D (7-AAD). These real-time methods have the advantage of excluding even small numbers of dead cells from analysis without the need for tedious separation procedures.[7,19,20]

It is important to note that during the early stages of apoptosis and necrosis, the size of cells decreases, either through a change in the refractive index of the cell (apoptosis) or through cell shrinkage (necrosis).[19] That is why the 3-part pre-differential leukocytes histogram is a helpful way to assess viability.

In the case of FNA gathering a large amount of apoptotic bodies (**Figure 3**), the WBC histogram will demonstrate a lymph peak that does not start at baseline with a mean event size lower than 30 fL. For aspirates that contain only viable cells (**Figure 1**), the lymph peak will start at baseline with size of cell larger than 35 fL. This method is a simple, fast and cost-effective way to screen cell viability, even if it is not so precise as the trypan blue or real-time methods

In conclusion, the pre-differentiation histogram of the lymphoid cells sampled by FNA is a helpful way to screen cell size of the lymphoid neoplasic population and adequacy of the aspirate specimen for flow cytometric analysis.

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