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review

Flow cytometric pitfalls in immunophenotyping of lymphomas

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Flow cytometry has been introduced as the most reliable method for the analysis of lymphocyte subsets. The advantage of flow cytometry is that it enables application of virtually all monoclonal antibodies (native cells), a quantitative estimation of different lymphoid cells, and that cells can be simultaneously analysed for multiple marker expression. The usage of flow cytometers requires considerable knowledge of physics and its technical application. Moreover, several problems arise from the complexity of the biological systems investigated.

Key words: antigens CD, flow cytometry, data processing, sample preparation, antibodies

Introduction

The recognition of lineages and definition of stage of lymphocyte differentiation permits an immunological approach to the classification of malignant lymphomas. A large panel of monoclonal antibodies is available for the identification of lymphocytes, their subsets, degree of differentiation and functional stages.[1] The most common criteria for establishing diagnosis of the lymphoma with the use of monoclonal antibodies is the demonstration of clonal expansion that may be demonstrated as a significant increase in proportion of a distinct lymphocyte subset, degree of maturation and functional stage.[2,3]

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Specimen collection

Cytological examination of the lymph node can be performed by fine needle aspiration biopsy, using a 23 Gx1 needle stacked several times through the same hole in different directions and at different levels of the lymph node. A hand made vacuum of 10 ml syringe is used for sample aspiration.

After the aspirate is taken, the needle is separated from the syringe for the first time in order to suck 1 ml of air into the syringe. The air is required to push a drop of sample on a glass slide for microscopic examination. Then the needle is disconnected for the second time, and attached to another syringe with 0.5 ml of PBS solution in it. A 3 ml EDTA Vacutainer tube is punctured by the

needle with the specimen. In this way the specimen is washed out from the needle by PBS solution.

Lymphocytes can also be obtained from extirpated lymph nodes. The dissociation of lymphocytes from lymphoid tissues is relatively easy to perform by teasing or forcing the tissue through the filter. The resulting cell suspension can be freed from debris by filtering of by allowing the debris to settle down. Cell suspensions from lymph nodes consists largely of lymphocytes and usually no erythrocyte lysis or density gradient centrifugation is required before labelling.

Contamination (blood or lipid particles) of the sample

Immunophenotyping of lymphoma usually requires multiparameter fluorescence analysis. An advantage of multiparameter analysis is better definition of cell phenotype, smaller quantity of sample (especially important in lymph node biopsy samples), and lower cost due to lower monoclonal antibodies consumption. For those reasons it is desirable to reserve all the available fluorescence colours for immune markers decisive for lymphoma cell definition. This means that lymphocytes should be, if possible, discriminated from other particles by light scatters only. A large numbers of non-lymphocyte particles in the sample can make it difficult to resolve lymphocytes. In lymph node aspirate, non-lymphocyte particles are most often represented by erythrocytes (due to blood contamination during sampling) or by lipid-particles - especially in abdominal lymph nodes sampling. In an attempt to solve these problems, two basic approaches for the blood contaminated samples may come into use.

The first approach is a density gradient separation, most often Ficoll-Hypaque density gradient. The cells that float on Ficoll-Hypaque are harvested. Density gradient centrifugation removes most erythrocytes, granulocytes and dead cells and lipid-particles as well. The disadvantage of the method is that it requires such a quantity of sample which is sometimes difficult to be provided by a fine needle biopsy. The method is also relatively time consuming.

Erythrocytes may also be removed by osmotic or detergent lysis (Figure 1). On the other had important blood contamination of the sample may also greatly influence determination of lymphocyte clonality (e.g. T/B ratio, kappa/gamma light chain ratio) because the sample is contaminated by blood lymphocytes that cannot be discriminated from lymph node lymphocytes. When erythrocytes are removed by lysis, blood-granulocytes remain in the sample and may serve as a good marker of blood contamination of the sample. Normally, no granulocytes are detected in lymph node aspirate. An important proportion of granulocytes in the sample indicate a strong blood contamination and therefore make the assessment of clonality unreliable.

The storage of samples before processing for the flow cytometer

Most authors agree that the best method of handling samples is keeping them anticoagulated at room temperature, diluted in an equal volume of tissue culture medium. Non-specific binding of immunoglobulin to Fc receptors on some lymphocyte subsets may be greatly augmented by exposure to cold. It is important to select a proper anticoagulant. Heparin, which has a strongly negative charge, may interfere with some later staining protocols. By contrast, anticoagulants that chelate calcium may be inactivated by the addition of tissue culture medium containing too much calcium. Under this condition, lymphocytes can be successfully analysed after a delay of about 24 hours.[2]

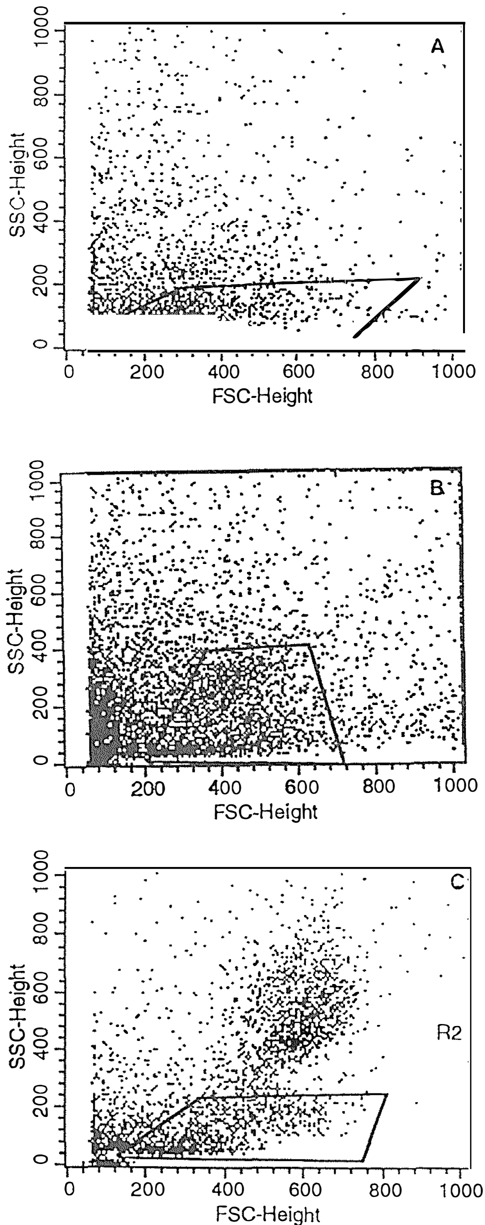

Figure 1. Normal lymph node aspirate (A) and blood contaminated lymph node aspirate - before (B) and after (C) detergent lysis of erithrocytes.

In immunophenotyping of lymphomas, it is often inconvenient to analyse cells on the flow cytometer immediately. Fixation of the cells allows samples to be processed later, after cytological and histological results are available. This could extremely reduce costs of immunophenotyping because only a few informative monoclonal antibodies are used instead of extensive panels. Additional markers may be determined on cryopreserved material; the procedure is effective, but unfortunately, rather time-consuming. Cells can also be fixed in formaldehyde. Cell pellets are resuspended in 2% formaldehyde and stored at 4°C in the dark. Fixed cells display similar light scatter signals as do native cells. However, labelling of formaldehyde-fixed cells may vary, compared to native or cryopreserved cells. Each antigen and monoclonal antibody should be first screened in order to verify eventual changes in labelling after fixation.

Labelling the samples

There are two major techniques for labelling the cells by single markers: direct, in which fluorochrome is directly attached to the antibody; and indirect, in which the marker is attached to a second step reagent which, in turn, binds to the antibody. Direct labelling is technically simpler; in very short time, it enables multi-colour (two, three, or four) labelling and, in general, results in less non-specific labelling. On the other hand, indirect labelling usually enables the attachment of several times more fluorochromes to each antigen and, therefore, obtains a brighter signal.[3]

It is important that only saturating amounts of antibodies are used during labelling. Self-prepared antibodies must therefore be titrated before used routinely and saturating dilution of antibodies must be determined. Manufactured antibodies are declared only for limited cell number. Especially in patients with lymphoma/leukaemia, cell numbers in the sample can easily exceed the declared cell numbers of the manufactur-

er. In that case, most antibodies are bond to cells and the effective concentration of antibodies is out of saturating range (Figure 2). The result is week labelling and cells can be presented as false negative.[4]

Washing of labelled cells is critical in indirect staining. The unbound first-step antibody must be removed completely before the second step. If not, some of the second-step antibody will bind to soluble first-step antibody and will not be available to bind to the cells. In addition, immune complexes formed de novo can bind non-specifically to several other cell types. Whenever indirect labelling is used with multiple antibodies, one must be very careful that the second-step reagents are specific in that they can distinguish the two primary antibodies. If two first step antibodies belong to different species, or are of different isotypes, it may be possible to use specific second-step antibodies that can distinguish them. For example, we can use rat and mouse first-step antibodies followed by anti-mouse and anti-rat specific immunoglobulins, or we can use IgG and IgM first-step antibodies followed by isotype specific second steps. The use of one antibody as a direct label and the second biotin-conjugated antibody is even better. The second-step reagent is avidin conjugated to the second fluorochrome.[5]

Washing at the end of the labelling procedure is important, especially if the cells are to be fixed. The usual fixation method is 1% - 2% paraformaldehyde. If soluble fluorochrome is present at the time of fixation, it may be fixed to the cell or may diffuse into the cell after fixation and lead to increased background.

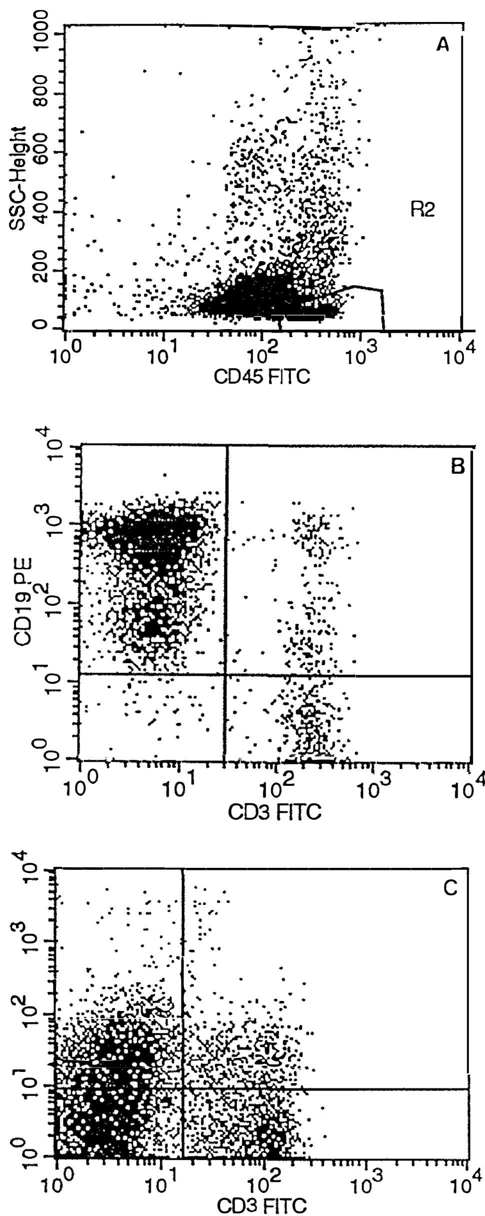

Figure 2. Insufficient CD19 Ab concentration. The fluorescence dott-plott (B) consists of CD3/CD19 negative cells followed by the cells that scatter into the positive CD19 range of the scale, but cannot be clearly separated from the negative population. Insufficient MoAb concentration may be due to low antibody titer, too many cells in the sample, pipetting problems or insufficient tube decanting before MoAb application. An addition of more CD19 Ab reveal a clearly CD19 positive cell population (A).

Selection of antibodies

The major advantage of monoclonal antibodies over polyclonal antibodies is cleaner labelling with less background. This is because polyclonal antibodies are used at 10

times higher concentrations or more. Therefore non-specific interactions and Fc receptor binding occur more often. $F(ab)_2$ fragments of polyclonal antibodies usually markedly decreases the tendency of antibodies to aggregate on storage and bind to cellular Fc receptors. The preparation of $F(ab)_2$ fragments of monoclonal antibodies is usually not necessary because they are used at low concentrations.

The labelling of antibodies must achieve a high specific labelling of immunoglobulin molecules and as low as possible concentration of nonlabelled fluorochrome in the preparation. FITC labelled antibody preparations are usually relatively free of the problems of non-specific labelling. On the other hand, rhodamine preparations of monoclonal antibodies may have relatively high concentration of free fluorochrome that cause non-specific binding.[6]

Another major problem in multiple colour analysis may be unbalanced intensity of monoclonal antibodies labelling. When FITC and rhodamine are used together, it is necessary to correct the red signal for the spill of fluorescein emission into the red region. This cannot be easy achieved if both fluorescence intensities are not balanced enough to successfully set the compensation. A problem can especially arise if we combine monoclonal antibodies of different manufacturers, or self-labelled antibodies with commercial antibodies, or polyclonal antibodies with monoclonal antibodies.

Non-specific fluorescence

In some preparations of conjugated antibodies, a lot of free (unbound) fluorescence dyes may persist. In that case, antigen discrimination may not be possible because most of the cells, especially monocytes, granulocytes and non-viable cells, exhibit fluorescence that is not linked to antigen expression. Antibodies must be purified (dialysis, gel chromatography) in order to get rid of unbound fluorescence dyes. It is advisable to inform the manufacturer about the unproper antibody preparation or simply - change it.

Some subsets of lymphocytes (macrophages, NK cells) have receptors bound to immunoglobulins by their Fc portion (Fc receptors). Therefore it is better to use the $F(ab)_2$ fragments of antibodies to decrease the non-specific binding, in particular when rabbit polyclonal antibodies are applied. $F(ab)_2$ fragments are also much more stable for long storage.[6]

Data analysis and gating methods

Lymphoma samples may not contain a homogenous population of malignant cells. Moreover, some types of lymphoma samples may include only a minor part of malignant cells, while the rest of cells represent the infiltration of non-malignant lymphocytes (mainly T cells). In such case, the detection of cell clonality is possible only after the malignant cells are first separated from normal cell infiltration and then analysed.

Many analysis tools in flow cytometry use the ability of the program to set gates on defining parameters. Only cells which fall within the gates are further analysed. For determination of cell clonality (e.g. Ig light chain restriction) a homogenous population of tumor cells may be first selected by setting gates on distinct parameters (e.g. cell size and granularity, CD19+, CD5+, CD23+ cells ...). Moreover, a gate can be set on a combination of selected parameters.

It is also possible to distinguish accurately lymphocytes from other leukocyte populations (bone marrow, peripheral blood) using the combination of fluorescence associated with CD45/CD14 and forward and orthogonal light scatter. By identifying the cell population of interest based on immunofluores-

cence, a light scattering window can then be drawn to include all of the lymphocytes. The combination of light scattering and immunofluorescence can also be used to define the purity of the gate. Once the optimal data acquisition gate has been established and characterized, it is possible to correct the subsequent analyses with that particular sample since the reactivity of monoclonal antibodies on monocytes and granulocytes can be accounted for once the nonlymphocytes have been identified as being within the acquisition gate.[7]

Reporting of data

The flow cytometric data are usually presented as: 1. percentages of positive cells 2. mean fluorescence intensities

ad.1. This operation requires that the positive cells be clearly resolved from the negative cells. In multicolour labelling, dot-blott diagrams must clearly demonstrate that a single, homogenous population is analysed. Otherwise, a calculation of "positive" cells by simply integrating a histogram data can be extremely misleading. For example, two different cell populations on fluorescence dotplot can represent as single, homogenous population on histogram.

ad.2. There are some instances in which it is not possible to resolve correctly the positive population. Some antigens are expressed in accordance to the physiological state of the cell. For example, there are some molecules, expressed on the cell surface during their activation (e.g. HLA-DR on T cells). Some other molecules are expressed constantly, but during cell activation, the level of their expression is increased (e.g. CD18, CD25). In the case of gradually expressed antigens, there is no sharp delineation between "positive" and "negative" cells. The use of isotype controls may be useful for cursor setting between "negative" and positive".

Conclusion

Flow cytometry is fast, easy, quantitative and reliable method for estimation of different lymphoid cells. The sample for flow-cytometric analysis must be prepared as a single cell suspension, hence, the loss of tissue structural information is inevitable. A flow-cytometry may therefore be of value as a method of subclassification of lymphoid cells observed in histological samples. Well selected panels of antibodies may provide a firm immunological basis for the classification, prognosis and treatment monitoring of lymphomas.

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