

Perspectives

Isotype Controls in the Analysis of Lymphocytes and CD34+ Stem and Progenitor Cells by Flow Cytometry—Time to Let Go!

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The isotype control has long been considered a useful part of both microscopic and flow cytometric immunologic assays, and, consequently, is still routinely used in clinical laboratories. In flow cytometry, the isotype control has traditionally been used to distinguish between fluorescent positive and fluorescent negative cell populations. Additionally, it has been used to estimate the number of cells reacting non-specifically with the target antibody under investigation. Over the past 10 years, the widespread use of directly conjugated monoclonal antibodies (mAb) and multiparameter analysis in clinical flow cytometry has reduced the need for a separate “negative control” tube. This tendency has materialized in guidelines recommending that the isotype control is irrelevant and potentially misleading in commonly used flow cytometric assays (3, 14). This perspective summarizes the rationale for omitting isotype control staining for surface membrane marker analysis, focusing on lymphocyte and CD34+ hematopoietic stem/progenitor cell analyses. Consequently, these points also pertain to the immunophenotyping of leukemia/lymphoma samples (14).

Prior to the development of directly conjugated mAb, pre-immune sera were used in microscopic and flow cytometric studies to estimate the level of “non-specific staining” of the specific antibody to its target cell, i.e., the binding of that specific antibody by mechanisms other than specific antibody-to-antigen interactions. Such non-specific binding is usually, but not exclusively, mediated by receptors that bind the Fc portion of the various immunoglobulin subclasses (19). In flow cytometry, an estimate of the number of cells reacting non-specifically is typically determined by placing a cursor at the foot of the isotype control negative population on a fluorescence (FL) histogram such that less than 2% of events are assessed as positive. This cursor position is maintained to determine the “percent positive cells” in the experimental stainings.

Currently, many isotype controls are produced by fusion of antibody producing cells with a myeloma-derived cell line to form a hybridoma. By the very nature of mAb production, antibodies produced by hybridomas will differ structurally from each other, even within the same immunoglobulin subclass or isotype. Thus mAb that “specifi-

cally” bind to the same antigen on the cells under study might each additionally bind “non-specifically” to other leukocytes, platelets, etc. in an unpredictable manner.

Other issues to consider in the use of monoclonal isotypes include differences in protein concentration and FL to protein (F/P) ratio between test antibody and isotype control. Different manufacturers use different protocols to produce, purify and chemically conjugate antibodies with a variety of fluorochromes which almost certainly impact the reliability with which experimental and isotype control mAb can be used to distinguish specific from non-specific binding. A compounding problem is that in a panel with several surface markers each would need their own isotype control matched for the above criteria. This is rarely done in the clinical laboratory.

SURFACE MEMBRANE STAINING OF LYMPHOCYTES

Centre for Disease Control (CDC) guidelines for the analysis of CD4+ T cells in HIV infected individuals (1) recommend that isotype controls should be used to determine the level of non-specific mAb binding to the cells and to set markers to distinguish FL negative from positive cells. In the analysis of lymphocytes, it is now feasible to simultaneously stain samples with 3 or even 4 directly conjugated antibodies, thus allowing Boolean gating to be employed to accurately identify subsets of interest while simultaneously excluding non-specifically stained cells. The latter usually include myelomonocytic cells that, in addition to exhibiting a higher level of autofluorescence than lymphocytes (11), express Type I, II and III Fc (IgG) receptors and are thus most likely to cause problems with non-specific staining (19). While the guidelines recognise that an isotype control is not needed if leukocyte populations are identified based on CD45 FL intensity (presumably when correlated with orthogonal light scatter [SSC]), they also state that isotype controls must be used when analysing populations that do not

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clearly resolve into positive and negative subsets, e.g., CD16+ or CD56+ lymphocytes. The recently published U.S.-Canadian Consensus recommendations on the immunophenotypic analysis of hematological neoplasia by flow cytometry state "a strong consensus that specific isotype controls provide no useful additional information beyond unstained cells alone, or negative cells in selected antibody combinations" (14). It is our opinion that these recommendations should also be adopted for lymphocyte subsets.

SURFACE MEMBRANE STAINING FOR RARE EVENT DETECTION

Enumeration of CD34+ cells in bone marrow, blood and apheresis products represents "rare event analysis" and presents some particular problems. For example, in single FL parameter analysis, the CD34 antibody used will often not only stain CD34+ cells specifically, but also other events non-specifically. Isotype control mAb are particularly inappropriate in this setting since they do not stain exactly the same number of events that are non-specifically stained by the CD34 antibody. Variation in staining of the same cell populations by different isotype controls has been reported (8, 17). Hence, multiparameter gating strategies were developed to reliably detect rare populations such as CD34+ stem and cells (2, 16, 17). This point is illustrated by the following examples. First, if the isotype control mAb detects lower non-specific binding than the CD34 mAb, its use to set the lower limit of the window of cell analysis for CD34+ cells leads to an overestimation of the number of CD34+ cells in a sample. This situation occurs in some cord blood and apheresis products that contain platelet aggregates non-specifically binding phycoerythrin (PE) conjugated CD34 mAb but not the fluorochrome and isotype matched control mAb from the same commercial source (18). In the second situation, the isotype control, even when appropriately titrated (17), stains more events non-specifically than are specifically stained by the CD34 antibody. This pattern can also be observed on samples containing dead and dying cells. In samples with suboptimal cell viability mAb may bind non-specifically to dead cells. Here, the exclusion of dead cells from the analysis using a viability dye such as 7-amino-actinomycin D (7-AAD) is the best approach (6, 10).

ALTERNATIVES TO ISOTYPE CONTROL MAB

Reviewing the many problems with isotype controls, why are most clinical laboratories still using them? The answer lies in the fact that we need something to compare our markers under investigation to. If negative and positive populations cannot be unambiguously resolved, how can we determine positive? This problem is particularly difficult to address when quantifying the expression of antigens exhibiting a continuous staining pattern ranging from negative to positive. Several approaches have been described to quantify populations with non-discrete FL signals. One is to report shifts in median or interquartile range of FL histogram channels of the population of interest (13) or to use a "binning" strategy, i.e., the

frequency distribution of data as a function of specific FL intensity intervals (5). Both strategies require standardised instrument setup and calibration of the instrument's response to FL signals to allow reproducible assessments of FL intensity. We will now review several alternative approaches some of which are likely to provide more useful information than conventional isotype control mAb.

ISOCLONIC CONTROLS

In an attempt to circumvent some of the problems outlined above, Coulter-Immunotech (Hialeah, FL and Marseille, France) have included an "isoclonic" control which consists of a 50-fold excess of unlabelled CD34 mAb relative to CD34 PE mAb in their Stem-Kit™ for progenitor cell enumeration. Thus, the excess unlabelled mAb blocks the specific staining of the conjugated mAb via its Fab part, thereby allowing the visualization of the non-specific binding of the CD34 mAb via its fluorochrome (PE). Although this type of control is thought by some to represent an improvement over conventional isotype and fluorochrome-matched controls (7), isoclonic controls will also block any Fc receptor mediated and other non-specific binding of the labelled CD34 antibody, thus underestimating background staining in the control as compared to the test sample. If conventional single FL parameter staining with the isoclonic control is used to set the discriminator between CD34 negative and positive cells, overestimation of the number of specifically stained cells may result.

As mentioned above, multiparameter gating using Boolean logic is one of the most useful methods of obviating the need for an isotype or isoclonic control in the analysis of rare cells (4, 17). In a recent study of 72 blood, apheresis and cord blood samples using the sequential gating strategy of the ISHAGE guidelines, which combine low SSC and intermediate forward light scatter with CD34 staining and dim CD45 expression (Fig. 1, panels 1 to 4), only 3 samples exhibited staining with the isoclonic control above 1 cell per micro litre (6).

BLOCKING WITH SERUM

Where high non-specific mAb binding of immunoglobulin is a problem, for example in some lymph node samples, one can first block Fc receptor-mediated and other non-specific binding by preincubating the cells in mouse or other serum. It has been suggested that the serum used should be from the same species as the antibody under investigation e.g., preincubate the sample with mouse serum when using mouse mAb (15).

UNSTAINED CELLS (AUTOFLUORESCENCE CONTROL)

The autofluorescence of unstained cells can be used as an initial reference to set FL markers to distinguish between negative from positive signals. Unstained cells are especially informative when autofluorescence is increased, e.g., due to treatment with anthracyclines (13). In the analysis of CD34+ cells, Owens and Loken recommend that the isotype control mAb must be titrated so that the negative cell population is matched to the unstained

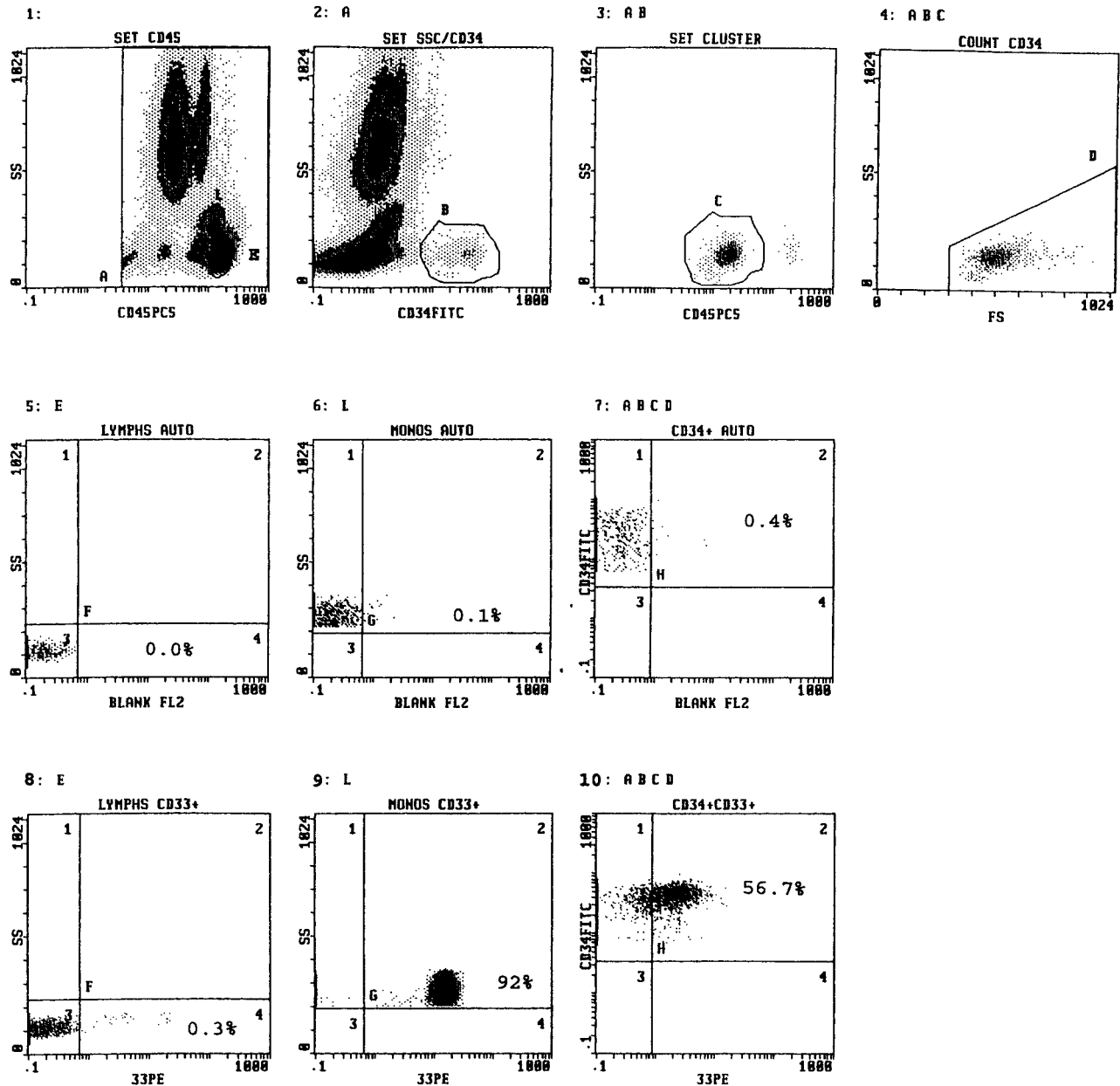


FIG. 1. Assessment of CD33 expression by CD34+ hematopoietic stem and progenitor cells. **Top row:** Panels 1 to 4 show a sequential Boolean gating strategy for the identification of stem/progenitor cells based on FSC, SSC and staining with CD34 FITC and CD45 PE-Cy5 (6, 17). In subsequent analyses, CD34+ cells are defined as cells meeting the criteria of Gates A, B, C and D. **Middle row:** Autofluorescence control. Sample stained with CD34 FITC and CD45 PE-Cy5. Autofluorescence on FL2 of lymphocytes (panel 5) and monocytes (panel 6) gated on regions E and L from panel 1, respectively. Panel 7 displays autofluorescence from CD34+ cells gated from region D on panel 4. **Bottom row:** Sample stained with CD34 FITC, CD33 PE and CD45 PE-Cy5. For convenience, the top four panels are displayed as common to both analyses. Panel 8 shows CD33PE on lymphocytes from panel 1. Panel 9 shows CD33+ monocytes gated from panel 1. Panel 10 shows CD34+, 33+ cells, with cursor position determined by autofluorescence from Panel 7.

autofluorescence control (9). In our opinion, this approach negates any utility of isotype control mAb-stained cells in comparison to unstained cells, particularly if this strategy results in isotype control mAb concentrations whose Fc receptor-mediated binding characteristics are no longer representative of the test antibody.

An alternative approach, proposed by the U.S.-Canadian Consensus recommendations (14), is to examine the negative populations within the investigated sample. For example, leukocytes stained for CD3 FITC and CD19 PE

will contain a population of lymphocytes that are negative for either marker. One can therefore visualise simultaneously the specific and nonspecific staining of the population of interest in one staining in comparison to unstained cells. While this works well for discrete markers, it cannot be applied to markers which show a continuous spectrum of reactivity from positive to negative, as is usually the case in the analysis of CD34+ cell subsets and activation markers on lymphocytes. In such cases alternative approaches can be used.

In the example of a panel of 3-color staining, e.g., CD3 FITC + CD19 PE + CD45 PE-Cy5 followed by CD3 FITC + CD16/CD56 PE + CD45 PE-Cy5, the lymphocytes will be negative for at least one antigen labelled with FITC or PE. The cursor can be set on this negative population and the subsequent tubes analysed at this cursor setting. Alternatively, when looking at subsets of CD34+ cells where the subsets are labelled with PE conjugates, a sample stained with only CD34 FITC and CD45 PE-Cy5 can be analyzed and the PE cursor placed on the autofluorescence of the accurately gated CD34+ cells. Subsequent stainings using mAb identifying the CD34 subsets of interest are then analyzed using the same CD34 gate and PE marker settings (Fig. 1, panels 7 and 10).

If none of the experimental markers yield a truly negative population on the cell subset of interest, the following approach can be used. We illustrate this approach using CD34+ cell subtyping on a sample stained with CD34 FITC, CD33 PE and CD45 PE-Cy5 (Fig. 1). Here, we use an alternative cell population within the sample, i.e., lymphocytes, which do not express the marker under study but have similar autofluorescence as the population of interest, i.e., CD34+ cells. First, the lymphocytes are gated on the basis of bright CD45 expression and low SSC, and the PE marker is placed on the lymphocytes which are CD33 negative (Fig. 1, panels 1 and 8). The expression of CD33 by the CD34+ cells is then analyzed with this PE marker setting.

ARE THERE STILL INDICATIONS FOR USING ISOTYPE CONTROL MAB?

There are still applications where the use of isotype controls is appropriate. When staining lymphocytes for intracellular markers it is important to monitor the effects of sample preparation, i.e. of fixation, permeabilization and washing on autofluorescence and non-specific mAb binding. If the fluorochrome-labelled antibody is trapped within the cell, but unbound to its target antigen, an autofluorescence control would underestimate FL signal resulting from non-specific mAb binding within the cell. With an isotype and conjugate-matched mAb control this problem will be detectable. Beyond the issue of sample processing, it is also important to select FITC-conjugated mAb with a low (ideally 1 : 1) fluorescein to protein ratio and PE conjugates with free PE removed. However, even in some cases of intracellular staining, isotype controls may be redundant. Consider the example of staining B lymphocyte precursors in a common acute lymphoblastic leukemia sample using surface CD45 expression and nuclear TdT. If there are residual TdT negative lymphocytes, this population can be identified by their bright CD45 expression compared to the dim CD45 expression on lymphoblasts and act as an internal negative control for TdT staining.

CONCLUSIONS

With an understanding of the limitations of isotypic controls and alternative methods which can be used to provide the necessary information, either by analysing

cellular/auto fluorescence, negative cell populations within the same test sample or Boolean gating for rare event analysis, the time has come to question the widespread use of isotype controls in flow cytometric immunophenotyping. While none of these methods are ideal they do, however, provide a reproducible reference point to determine negative from positive staining. Let us move once and for all to adopt these more meaningful methods of analysis.

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