Hematopathology / Flow Cytometric Analysis of Clonal Plasma Cells

Single Tube, Six-Color Flow Cytometric Analysis Is a Sensitive and Cost-Effective Technique for Assaying Clonal Plasma Cells

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Abstract

Bone marrow flow cytometric analysis is a powerful and rapid tool for evaluating plasma cell myeloma. By using a noncontrolled patient population in various stages of diagnosis and treatment, we compared 6-color (single-tube) and 4-color (multiple-tube) flow cytometric immunophenotyping protocols. Prospective comparison in 52 cases demonstrated improved ability to detect clonal plasma cells or identical diagnoses in 100% of the cases using 6-color, single-tube analysis. In cases in which 6-color flow cytometric analysis improved detection of a clonal population, concurrent biopsy showed less than 5% involvement by plasma cell myeloma, suggesting that 6-color flow cytometry has an advantage in patients with a low disease burden. In addition, the simplification of the procedure resulted in substantial savings in technologist time and reagent costs. Taken together, this study demonstrates that 6-color flow cytometry is an excellent, cost-effective means to assay for clonal plasma cells in a noncontrolled patient population.

Plasma cell neoplasms are defined by a neoplastic clonal population of mature plasma cells. According to criteria from the International Myeloma Working Group and the 2008 World Health Organization Classification of Tumours of Hematopoietic and Lymphoid Tissues, these entities are subdivided into monoclonal gammopathy of undetermined significance, plasma cell myeloma, extramedullary plasmacytoma, and monoclonal immunoglobulin depositing diseases based on several criteria, including the presence of a serum or urine M spike, organ or tissue damage, and demonstration of a clonal plasma cell population. A minimal percentage of abnormal plasma cells is not strictly required for a diagnosis of symptomatic plasma cell myeloma.

Bone marrow flow cytometric analysis is a powerful and rapid tool for evaluating plasma cell clonality. Nonneoplastic plasma cells are characterized by high levels of surface expression of CD19, CD38, and CD138 and dim expression of CD45. In contrast, neoplastic plasma cells typically show loss of CD19 and aberrant expression of markers such as CD56 and CD117. In addition, immunoglobulin heavy and light chain staining can also be assayed to assess for plasma cell clonality.

The assessment of light chain clonality is complicated by the cytoplasmic localization of immunoglobulins in plasma cells, requiring that they be permeabilized before determining the immunoglobulin profile. In 4-color analysis, the combination of numerous surface and cytoplasmic markers requires separate protocols and multiple tubes for analysis. In addition to increasing the chances of error, the presence of multiple tubes and protocols results in increased cost of the assay in terms of reagents and technician hours.

With the advent of 6- and 8-color analysis, the use of single-tube flow cytometric analysis for plasma cell myeloma...
has been examined. Single-tube, 6-color flow cytometric analysis has been shown to be more sensitive than 4-color analysis combined with immunofluorescence\(^6\) and as effective as existing 3-color methods for detecting minimal residual disease.\(^7\) The latter comparison was performed in a limited patient population at day 100 following therapy. Thus, although encouraging, the evaluation of 6-color analysis in studies was limited by comparison with protocols with un common techniques or limited patient populations that may increase their sensitivity. A comparison of standard, conventional 3- or 4-color and single-tube, 6-color methods in a more variable clinical setting has not yet been reported.

To determine the feasibility of using a single-tube, 6-color method, we conducted a prospective comparison of a multiple tube 4-color gating method with a single tube, 6-color strategy using samples from our patients in routine clinical practice in a tertiary care center. The use of a 6-color method resulted in increased sensitivity of detection of clonal plasma cell populations and reduced cost in terms of reagents and technologist time. We concluded that single-tube, 6-color flow cytometry is a broadly applicable, sensitive, and cost-effective method for assaying the presence of clonal plasma cell populations.

Materials and Methods

We analyzed samples from 52 consecutive patients undergoing flow cytometric analysis for clonal plasma cell populations using 4- and 6-color methods. Four-color flow cytometry was performed by standard clinical procedures, and results were analyzed by hematopathologists not involved with the study. Following this standard clinical analysis, an additional single-tube, 6-color analysis was performed, the results of which we analyzed independent of the 4-color analysis. The clinical data were recorded, including sex, age, preexisting plasma cell neoplasms, and therapy.

The monoclonal antibody combinations for the 4-color analysis were CD56/CD38/CD19/CD138; surface \(k/\lambda\)/cytoplasmic \(\lambda/\lambda\); and no antibody/no antibody/CD19 (permeabilization control tube) corresponding to fluorescein isothiocyanate (FITC)/phycoerythrin (PE)/peridinin chlorophyll protein (PerCP)/allophycocyanin (APC) fluorochromes. The 6-color tube contained cytoplasmic \(k/\lambda\)/CD138/CD38/CD56/CD19, corresponding to FITC/PE/PerCP-Cy5.5/PE-Cy7/APC/APC-H7 fluorochromes. All antibodies were purchased from Becton Dickinson Biosciences, San Jose, CA. Fixation and permeabilization were performed using the Fix and Perm reagent system (Invitrogen, Carlsbad, CA). For both methods, 20 \(\mu\)L of antibody was added for FITC, PE, PerCP, and PerCP-Cy5.5 reagents, and 5 \(\mu\)L of reagent was added for APC, APC-H7, PE, and PE-Cy7.

Samples analyzed using the 4-color method were first washed in isotonic solution. For surface analysis (tubes 1 and 2), antibodies were added and incubated in the dark for 15 minutes. Next, 100 \(\mu\)L of 1:10 BD FACS Lysing Solution (BD Biosciences, San Jose, CA) was added for 15 minutes, and the sample was spun at 2,500 rpm for 5 minutes. The specimen was then decanted, and 0.5 mL of deionized water was added for 5 minutes before data acquisition. For cytoplasmic studies (tubes 3 and 4), CD19 was first added for 15 minutes and then washed with 1 mL of isotonic solution and centrifuged for 5 minutes at 2,500 rpm. The specimen was decanted and then fixed and permeabilized, followed by addition of \(k/\lambda\) antibodies or isotype controls according to the manufacturer’s instructions.

For samples analyzed using the 6-color method, surface antibodies (CD138, CD38, CD56, and CD19) were added and incubated for 15 minutes in the dark. The sample was washed in isotonic solution, centrifuged, decanted, and fixed and permeabilized, followed by addition of \(k/\lambda\) antibodies or isotype controls according to the manufacturer’s instructions.

Analysis was performed using a BD FACSCanto II flow cytometer with FACSDiva Software (BD Biosciences). The total number of cells analyzed in both methods varied based on the sample size and concentration but was generally greater than 100,000 cells. For cases analyzed by the 4-color method, the average number of cells counted was 94,548 (range, 6,640-324,961). For 6-color analysis, the mean number of cells counted was 140,287 (range, 15,203-300,000). Forward and side scatter characteristics were used for plasma cell gating in the 4-color analysis. Side scatter and high-level CD38 expression were used for plasma cell gating in the 6-color analysis. A sample was considered positive if a cluster of plasma cells with monotypic cytoplasmic immunoglobulin staining could be identified with or without CD56 expression or if a CD56\(^+\) plasma cell population was identified with a cytoplasmic immunoglobulin \(k/\lambda\) ratio greater than 4:1 or less than 1:2. A result was considered “suspicous” if a population of CD56\(^+\) plasma cells was present with a cytoplasmic immunoglobulin \(k/\lambda\) ratio less than 4:1 or greater than 1:2 or if a CD56\(^−\) plasma cell population had a cytoplasmic immunoglobulin \(k/\lambda\) ratio greater than 4:1 or less than 1:2. Finally, a negative interpretation was made if the cytoplasmic immunoglobulin \(k/\lambda\) ratio was less than 4:1 or greater than 1:2 in the absence of CD56\(^+\) plasma cells.

Results

Flow cytometric analysis was performed on 52 consecutive bone marrow aspirates in patients with a known or suspected plasma cell neoplasm. Of the patients, 41 (79%) had a previous diagnosis of a plasma cell neoplasm; however, only
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4 of these patients had previously undergone autologous stem cell transplantation. Of the patients, 37 were men and 15 were women. The average age was 61 years.

Of the 52 cases studied, 44 (85%) had identical interpretations using 4- and 6-color methods. Of these cases, 32 were positive for a monoclonal plasma cell population (73%), 10 were negative (23%), and 2 were suspicious (5%). The results are summarized in Table 1.

In the remaining 8 cases (15%), we were able to improve our ability to detect a clonal plasma cell population by using the 6-color flow cytometric method. Two cases were called suspicious for the presence of a clonal plasma cell population using 4-color analysis but were interpreted as positive using 6-color analysis as a result of an abnormal cytoplasmic immunoglobulin \( \kappa/\lambda \) ratio as defined in the “Materials and Methods” section. Three cases were adjusted from negative to suspicious for the presence of a clonal plasma cell population, based on CD56 expression in 1 case and an abnormal cytoplasmic immunoglobulin \( \kappa/\lambda \) ratio in the others, and the interpretations in 3 cases went from negative to positive after 6-color analysis, based on CD56 expression and an abnormal cytoplasmic immunoglobulin \( \kappa/\lambda \) ratio in all cases. Comparison of the 4- vs 6-color flow cytometric results using the Fisher exact test indicated that the difference in results was statistically significant (\( P < .0001 \), comparison of positive and suspicious results vs negative results).

The mean and range of total cells analyzed in each method are listed in Table 2, and the mean and range of cells gated in each method are listed in Table 3. Overall, a higher number of total cells were analyzed using 6-color analysis compared with 4-color analysis. Furthermore, this trend of counting a higher total number of cells in 6-color analysis was present in the subset of cases in which use of 6 colors improved detection of clonal plasma cells and those in which detection was not improved (Table 2), indicating that the increased sensitivity of the 6-color method cannot be attributed solely to a higher number of total cells counted.

### Table 1

Comparison of Four- and Six-Color Flow Cytometric Immunophenotypic Analysis to Detect Clonal Plasma Cell Populations

<table>
<thead>
<tr>
<th>6-Color Analysis</th>
<th>Positive</th>
<th>Suspicious</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>32 (62)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Suspicious</td>
<td>2 (4)</td>
<td>2 (4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Negative</td>
<td>3 (6)</td>
<td>3 (6)</td>
<td>10 (19)</td>
</tr>
</tbody>
</table>

* Data are given as number (percentage). \( P < .0001 \); Fisher exact test, comparison of positive + suspicious results vs negative results.
contrast, the number of gated cells in 4- and 6-color methods differed between cases in which 6-color analysis improved detection of clonal plasma cells compared with those in which detection was not improved (Table 3). In cases in which 6-color analysis improved the ability to detect a clonal plasma cell population, fewer gated cells were analyzed compared with the 4-color technique. Taken together, these data support the finding that 6-color, single-tube analysis is a more sensitive technique for detecting clonal plasma cell populations than the 4-color multi-tube technique.

Concurrent bone marrow biopsies were performed for all cases. In the cases that were interpreted as positive using both

**Image 2** Example of a case interpreted as “suspicious” for a plasma cell neoplasm by 4-color analysis (A) and positive by 6-color analysis (B). Gating was performed as in Image 1. The CD38+ plasma cell population (circled) was positive for CD56 in both methods (second row). Monotypic immunoglobulin λ light chain expression was seen in the 6-color analysis but was not detected using 4-color analysis (fourth row). APC, allophycocyanin; C, cytoplasmic; Cy, cyan; PE, phycoerythrin; PerCP, peridinin chlorophyll protein.

**Image 3** Example of a case interpreted as negative for a plasma cell neoplasm by 4-color analysis (A) and positive by 6-color analysis (B). Gating was performed as in Image 1. The CD38+/CD56+ plasma cell population (circled) was only detected using 6-color analysis. The aberrant plasma cell population was positive for CD56 (second row) with monotypic immunoglobulin λ light chain expression (third and fourth rows) by 6-color analysis but not by 4-color analysis. APC, allophycocyanin; C, cytoplasmic; Cy, cyan; PE, phycoerythrin; PerCP, peridinin chlorophyll protein.
4- and 6-color methods, the percentage of monotypic plasma cells in concurrent bone marrow biopsy specimens ranged from 5% to 90%, with an average of 35% involvement. In contrast, in the 8 cases in which 6-color flow cytometry improved our ability to detect a clonal population, the concurrent biopsy specimens showed only a small population of plasma cells, with 7 cases having 5% plasma cells or fewer and 1 case with approximately 20% plasma cells. Thus, 6-color flow cytometry has an advantage over the 4-color method for detecting clonal plasma cells in patients with a low percentage of bone marrow involvement.

The 6-color method resulted in substantially reduced costs in labor and materials. The average time for a technician to perform the analysis was reduced by approximately 24 minutes using the 6-color method, a cost savings of approximately $15. In addition, the use of fewer reagents, including antibodies and the fixation/permeabilization solutions, yielded a savings of $31.32 per patient. In our center, we performed approximately 470 flow cytometric analyses for plasma cells in the past year, which would have reduced reagent costs by approximately $14,700 and labor costs by $7,050.

**Discussion**

In this study, we compared 4- and 6-color flow cytometric protocols for the ability to detect clonal plasma cell populations and for cost-effectiveness. In all cases, the use of the 6-color method resulted in identical diagnoses or improved our ability to detect clonal plasma cells. In addition, the simplification of the procedure results in an estimated yearly savings of $7,050 in labor costs and an estimated yearly savings of $14,700 in reagent costs. Taken together, these data demonstrate that 6-color flow cytometry is an excellent, cost-effective means to assay for clonal plasma cells.

Several methods can be used to assay for plasma cell clonality, including the presence of aberrant surface marker expression and clonal cytoplasmic immunoglobulin expression. Although analysis of aberrant surface marker expression is technically less cumbersome owing to the lack of permeabilization, a study by Cao et al detected a change in surface marker phenotype in 41% of 56 cases examined, including expression of CD56, CD20, and CD52. In contrast, no change was seen in the cytoplasmic immunoglobulin light chain. Their report highlights the importance of using a method that assays light chain expression to improve the sensitivity of the analysis for the presence of clonal plasma cells.

Recently, it has been shown in a prospective study that minimal residual disease at 100 days after transplantation was the most important prognostic factor in patients with multiple myeloma. Although this study demonstrates the importance of evaluating plasma cell populations by flow cytometry, the design of the study contained several parameters that limit its application to daily practice. The patients in the study underwent flow cytometric analysis at the time of initial diagnosis with documentation of an aberrant surface phenotype, but immunoglobulin light chain expression was not used in the flow cytometric analysis. In addition, the patient population was studied at a uniform time point after transplantation (100 days). Using these 2 conditions will likely be challenging outside a controlled research study environment. For example, at our tertiary referral center, diagnoses are often made years previously at outside institutions, and initial aberrant phenotypes may not have been measured or documented. In addition, many of the patients may have undergone transplantation.
previously without the possibility for flow cytometric analysis at the designated time points. Thus, despite the promising results of the study by Paiva et al., alternative methods are still essential for monitoring clonal plasma cell populations independent of controlled studies of minimal residual disease.

An additional advantage of single-tube flow cytometric analysis is reduction in the chance for technologist error. Standard 4-color analysis involves multiple tubes, with different antibodies, a subset of which must undergo permeabilization. This complexity in procedural requirements increases the chances of error, whereas a single-tube method is far simpler and will likely reduce the chance for mistakes. In addition to improving the quality of patient care, preventing errors using this streamlined approach will save technologist time and effort.

Previously, single-tube, 6-color flow cytometric analysis was shown to be as effective as existing 3-color methods; however, the study was performed in a controlled patient population following induction chemotherapy and at day 100 following consolidation. The authors of the study noted that the comparison may be limited owing to artificially increased sensitivity in the 3-color studies related to the relative paucity of confounding abnormal plasma cells following treatment. As such, extrapolation from that study may not be applicable to standard flow cytometric protocols in a less-controlled, more clinically diverse patient population. In the present study, we prospectively compared 4- and 6-color methods in patients with plasma cell myeloma in a variety of clinical settings. Our results suggest that a single-tube, 6-color method will likely have broad applicability in a more varied patient population.

In 2008, the European Myeloma Network issued guidelines for flow cytometric analysis. Their recommendations highlight the role of flow cytometry in distinguishing between reactive and neoplastic plasma cell populations and include use of CD138, CD38, and CD45 in a single tube, as well as κ and λ immunoglobulin light chain expression to demonstrate clonality. The minimal antigens recommended for assessing abnormal markers are CD19 and CD56. With the exception of CD45, our technique conforms to these recommendations. In addition, the relative simplicity of the single-tube, 6-color technique would permit the use of a supplementary tube containing additional markers such as CD45, CD20, or CD117, if necessary.

One of the major limitations of flow cytometric analysis for plasma cell neoplasms is the inability to accurately quantify the percentage of clonal plasma cells in the marrow. The 2008 World Health Organization diagnostic criteria for plasma cell myeloma include several changes, 2 of which have particular relevance to the role of flow cytometry. First, the minimal percentage of plasma cells required for a diagnosis of plasma cell myeloma has been lowered from 30% to 10%. Second, a minimal percentage of abnormal plasma cells is no longer required for a diagnosis of symptomatic plasma cell myeloma. The most significant diagnostic criteria for this entity are the presence of a monoclonal serum or urine protein and organ or tissue damage. Although the guidelines state that monoclonal plasma cells will usually exceed 10%, approximately 5% symptomatic myeloma cases will have fewer than 10% plasma cells. Thus, although quantitation of abnormal plasma cells by bone marrow biopsy and aspirate remains the “gold standard” for assaying disease burden, it is likely that flow cytometric analysis will have an increasingly important role in diagnosing patients with plasma cell myeloma. Our study shows that single-tube, 6-color flow cytometric analysis is a more sensitive, more cost-effective, and, likely, less error-prone technique than standard multiple-tube, 4-color analysis in a noncontrolled patient population with plasma cell myeloma.

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References