Four-Color Flow Cytometric Analysis of Myeloma Plasma Cells

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Key Words: Multiple myeloma; Four-color flow cytometric analysis; Immunofixation

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Abstract

We monitored the behavior of residual myeloma plasma cells in patients with multiple myeloma after high-dose therapy and autologous or allogeneic transplantation using 3 methods of a flow cytometric technique using 4-color staining, immunofixation, and polymerase chain reaction approaches. We analyzed 17 cases by a relatively simple flow cytometric technique using CD38/CD45/CD19/CD56. Detectable myeloma plasma cells were found in 5 patients at diagnosis and 9 patients after treatment. Of 14 cases, 9 (64%) had CD19–CD56+ myeloma plasma cells, and 5 (36%) of 14 had CD19–CD56− myeloma plasma cells. When 37 bone marrow samples that had less than 5% myeloma plasma cells were assessed, myeloma plasma cells were detected in all 20 immunofixation-positive cases and 3 of 17 immunofixation-negative cases (P = .002). All 4 polymerase chain reaction–negative samples characterized as immunofixation-negative contained no detectable myeloma plasma cells. Flow cytometry can provide effective information to detect low levels of myeloma plasma cells.

Many patients who receive high-dose therapy and autologous or allogeneic transplantation for myeloma undergo complete remission by conventional criteria, with a minority achieving a molecular remission. Complete remission is defined as the absence of the original monoclonal paraprotein in serum and urine by immunofixation and fewer than 5% plasma cells in the bone marrow (BM).1 Effective maintenance strategies require more sensitive methods for the detection of low-level disease. Polymerase chain reaction (PCR) assay methods using primers specific for the neoplastic variable-diversity-joining (VDJ) region permit sensitivities of up to 1 in 10^5 cells if no other B cells are present. If B cells are present, which is the case in most patient samples, the assay will identify a population that represents more than 2% of total amplifiable B cells, equating to a sensitivity of 1 in 10^3 to 1 in 10^4 to total leukocytes.2,3 Such approaches are not quantitative and are labor-intensive. Furthermore, only 60% to 70% of patients have an amplifiable VDJ region.4 Studies using 3-color flow cytometry have demonstrated that a flow cytometric assay can detect 1 plasma cell in 10^4 leukocytes.3,5 Plasma cell phenotypic aberrations by flow cytometry using a panel of monoclonal antibodies in quadruple combinations (CD38/CD56/CD19/CD45, CD138/CD28/CD33/CD38, and CD20/CD117/CD138/CD38) were identified at diagnosis and then used as patient-specific probes for follow-up analyses.6 We monitored the behavior of residual myeloma plasma cells in patients with multiple myeloma (MM) after high-dose therapy and autologous or allogeneic transplantation using 3 different methods of a flow cytometric technique using 4-color staining against CD38/CD45/CD19/CD56, immunofixation, and PCR.
Materials and Methods

Cases
The 17 cases included in this study were analyzed at the Kanagawa Cancer Center, Yokohama, Japan, between July 2003 and March 2005. Cases 1 through 5 were new and untreated at sampling, but cases 6 through 17 were treated at sampling. The median age at diagnosis was 61 years (range, 34-73 years), with a male/female ratio of 9:8.

All patients were treated with conventional chemotherapy with vincristine, doxorubicin (Adriamycin), and dexamethasone (VAD), followed by collection of peripheral blood stem cells with high-dose etoposide (500 mg/m^2) for 3 days and a subsequent course of peripheral blood stem cell–supported high-dose melphalan (200 mg/m^2). A second autologous transplantation was followed within 3 to 6 months using the same chemotherapy regimen. Response was monitored by means of serum electrophoresis and immunofixation, which was performed on serum. Designation of a complete response required fewer than 5% plasma cells in the BM aspirate and negative serum electrophoresis and immunofixation results.

For standardization procedures, we used BM samples from 10 acute myelogenous leukemia, 5 chronic myelogenous leukemia, 2 severe aplastic anemia, and 1 acute lymphoblastic leukemia cases after transplantation as negative control samples without MM. Informed consent was obtained from all patients before performing these procedures.

Flow Cytometry

Cell Surface Staining
Leukocytes were prepared by incubation with a 10-fold excess of ammonium chloride (8.6 g/L in distilled water) for 15 minutes and washed twice with phosphate-buffered saline with 0.5% bovine serum albumin (BSA). Then, 1 x 10^6 leukocytes were stained with each of the following monoclonal antibody conjugates for 30 minutes at 4°C: CD38 fluorescein isothiocyanate (FITC); CD45-phycocerythrin/cyanin 5.1 (PC5); CD19-phycocerythrin/cyanin 7 (PC7); and CD3-phycocerythrin (PE), CD56-PE, or CD138-PE (Immunotech, Marseille, France). Cells were washed twice with 0.5% BSA and analyzed by means of an Epics XL with EXPO 32 software (Beckman Coulter, Fullerton, CA). Between 200,000 and 500,000 total cells were analyzed in each test.

The gating strategy was optimized to exclude contaminating events, particularly apoptotic cells and cellular debris. Analysis of CD38 vs CD45 expression (left) provides the best separation of plasma cells from other leukocytes. The CD38+ plasma cells in each case showed negative to strong positive variable staining for CD45. Thus, an initial region (R1) is set around a large number of cells expressing a high level of CD38 and a high level, a low level, or lack of CD45 in each case (Image 1A, left). A second region (R2) is set on the forward scatter vs side scatter to exclude apoptotic cells and cellular debris (Image 1A, center). A third region (R3) is set on the side scatter vs CD38 to include all CD38+ leukocytes (Image 1A, right). The plasma cell population satisfies all of R1, R2, and R3. IgG1 PC7 and IgG1 PE (Immunotech) served as negative controls.

Horizontal quadrant markers were set according to the CD3 control for analysis of CD56 and CD138 expression. The expression of CD19-PC7 and CD56-PE was used to distinguish between normal and neoplastic plasma cells. The former are consistently CD19+CD56– or CD56dim, whereas

Table 1
Characteristics of and Results for 17 Patients With Multiple Myeloma

<table>
<thead>
<tr>
<th>Case No./ Sex/Age (y)</th>
<th>M Protein</th>
<th>Time of Study</th>
<th>IEP</th>
<th>Plasma Cells in BM Smears</th>
<th>Myeloma Cells: CD38, CD45 Gating (%)</th>
<th>CD19/CD56</th>
<th>CD138 (%)</th>
<th>β_2-MG</th>
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<tr>
<td>1/M/40</td>
<td>IgA-λ</td>
<td>Diagnosis</td>
<td>IgA-λ</td>
<td>50.0</td>
<td>35.803 –/+</td>
<td>55.0</td>
<td>3.1</td>
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</tr>
<tr>
<td>2/F/61</td>
<td>IgG-λ</td>
<td>Diagnosis</td>
<td>IgG-λ, BJP-λ</td>
<td>56.2</td>
<td>60.434 +/+</td>
<td>88.7</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>3/F/63</td>
<td>BJP-λ</td>
<td>Diagnosis</td>
<td>BJP-λ</td>
<td>20.4</td>
<td>16.461 +/+</td>
<td>48.0</td>
<td>12.6</td>
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<td>4/F/68</td>
<td>BJP-λ</td>
<td>Diagnosis</td>
<td>BJP-λ</td>
<td>37.0</td>
<td>30.906 +/+</td>
<td>81.0</td>
<td>2.2</td>
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<tr>
<td>5/M/73</td>
<td>BJP-κ</td>
<td>Diagnosis</td>
<td>BJP-κ</td>
<td>74.0</td>
<td>76.386 +/+</td>
<td>35.0</td>
<td>ND</td>
<td></td>
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<tr>
<td>6/M/26</td>
<td>IgG-κ</td>
<td>PC</td>
<td>IgG-κ</td>
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<td>22.291 +/+</td>
<td>15.0</td>
<td>ND</td>
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<tr>
<td>7/M/62</td>
<td>IgG-κ</td>
<td>PC</td>
<td>IgG-κ</td>
<td>2.8</td>
<td>1.429 +/+</td>
<td>72.9</td>
<td>1.8</td>
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<tr>
<td>8/M/65</td>
<td>IgAκ</td>
<td>PC</td>
<td>IgAκ</td>
<td>7.0</td>
<td>3.747 +/+</td>
<td>57.4</td>
<td>ND</td>
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<td>9/M/73</td>
<td>IgGκ</td>
<td>PC</td>
<td>IgGκ, BJPκ</td>
<td>51.6</td>
<td>42.267 +/+</td>
<td>60.0</td>
<td>9.7</td>
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<tr>
<td>10/F/54</td>
<td>IgGκ</td>
<td>PC</td>
<td>IgGκ</td>
<td>1.8</td>
<td>0.235 +/+</td>
<td>20.0</td>
<td>1.5</td>
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<tr>
<td>11/M/47</td>
<td>IgAκ</td>
<td>PC</td>
<td>–/+ IFE- –/+ PCR-</td>
<td>0.6</td>
<td>0.000 ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12/F/50</td>
<td>IgGκ</td>
<td>PA PBSCT</td>
<td>IgGκ</td>
<td>17.6</td>
<td>13.924 –(+ at diagnosis)</td>
<td>85.5</td>
<td>4.7</td>
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<tr>
<td>13/F/62</td>
<td>IgGκ</td>
<td>PA PBSCT</td>
<td>IgGκ</td>
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<td>7.670 –/+</td>
<td>79.4</td>
<td>4.7</td>
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<tr>
<td>14/F/61</td>
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<td>PA PBSCT</td>
<td>–/+ IFE-</td>
<td>1.0</td>
<td>0.000 ND</td>
<td>1.2</td>
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<td></td>
</tr>
<tr>
<td>15/M/24</td>
<td>IgDκ</td>
<td>PA PBSCT</td>
<td>IFE-</td>
<td>0.0</td>
<td>0.043 +/+</td>
<td>6.0</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>16/F/53</td>
<td>IgAκ</td>
<td>PA PBSCT</td>
<td>–/+ IFE-</td>
<td>0.8</td>
<td>0.032 ND</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17/F/59</td>
<td>IgDκ</td>
<td>PA PBSCT</td>
<td>–/+ IFE-</td>
<td>0.4</td>
<td>0.032 ND</td>
<td>8.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

β_2-MG, β₂-microglobulin; BJP, Bence Jones protein; BM, bone marrow; IEP, immunoelectrophoresis; IFE, immunofixation electrophoresis; ND, not done; PA, postautologeneic; PAU, postautologous; PBSCT, peripheral blood stem cell transplantation; PC, postchemotherapy; PCR, polymerase chain reaction; +, positive; –, negative.

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the latter are CD19– or CD19+CD56+.³⁹ Myeloma plasma cells express higher levels of CD138 than normal plasma cells.³ The expression of CD138-PE is useful for making it possible to discriminate between the majority of plasma cells and other hematopoietic cells (particularly B-progenitor cells) Image 1B.¹⁰ The level of CD19 expression is broad on normal plasma cells, and up to 26.0% may be CD19– compared with negative control samples. Therefore, samples were classified

Image 1 Flow cytometric analysis of myeloma plasma cells. A, The gating strategy used to detect plasma cells. B, Top row, case 2 at diagnosis with only neoplastic phenotype (CD19–CD56+) cells. Middle row, a case of severe aplastic anemia after transplantation with only normal phenotype (CD19+CD56–) plasma cells. Bottom row, case 12 at 31 months after transplantation with neoplastic phenotype (CD19–CD56– or CD56+) and normal phenotype (CD19+CD56–) plasma cells. PC7, PE/cyanin 7; PE, phycoerythrin.
as containing neoplastic plasma cells only if more than 26.0% had an abnormal phenotype. A minimum of 50 events that satisfied the gating strategy (Image 1) were required for identification of a neoplastic or normal plasma cell population. Up to 500,000 events were acquired, allowing a maximum sensitivity of detection of 1 plasma cell in 10⁴ leukocytes.³⁵

**Cytoplasmic Immunoglobulin Staining**

A combination of CD38-PE, CD45-PC5, and cytoplasmic immunoglobulin staining was performed. Cells were incubated with CD38-PE and CD45-PC5 (Immunotech) for 30 minutes at 4°C. Cells were exposed to the reagents using the Intrastain Kit (DAKO, Glostrup, Denmark), according to the manufacturer’s instructions. The permeabilized cells then were incubated with anti–κ-FITC, anti–λ-FITC, anti–IgA-FITC, anti–IgD-FITC, anti–IgG-FITC, anti–IgM-FITC, or isotype-negative control antibody for 30 minutes at 4°C. Cells were washed with 0.5% BSA, and data were acquired by means of the Epics XL with EXPO 32 software. A total of 25,000 cells was analyzed in each test Image 2ll.

**PCR Amplification**

Immunoglobulin heavy chain PCR was performed at Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo, Japan.

**Image 2** (Case 1) Flow cytometric analysis of cytoplasmic immunoglobulin light and heavy chain expression diagnosis using 3-color staining. The dot plot shows CD38+CD45+ cells that were monoclonal for λ cytoplasmic light chain and IgA cytoplasmic heavy chain. FITC, fluorescein isothiocyanate; PC5, PE/cyanin 5.1; PE, phycoerythrin.
High-molecular-weight DNA was obtained from separated leukocytes using the DNAzol reagents (Invitrogen, Carlsbad, CA). DNA was amplified with a primer to a consensus region of the J heavy chain (JH) gene and a primer to a consensus framework 2 region or a primer to a consensus framework 3 region, as previously described. Products were then electrophoresed and analyzed using an ABI automated DNA sequencer PRISM 3100 Genetic Analyzer (Applied Biosystems, Cheshire, England).

### Statistical Analysis
Statistical analyses were carried out using the Student t-test.

### Results
The CD38+ plasma cells in each case showed negative to strong positive variable staining for CD45 (Images 1 and 2). Myeloma plasma cells can be distinguished from normal plasma cells on the basis of CD19 and CD56 expression (Image 1). Of 17 cases of MM, myeloma plasma cells were detected by flow cytometry in 5 at diagnosis and 9 of 12 patients after treatment. All myeloma plasma cells from the 5 patients assessed at diagnosis showed their phenotype to be CD19–CD56+. After treatment, 5 (56%) of 9 patients had CD19–CD56– myeloma plasma cells, and 4 (44%) had CD19–CD56+ myeloma plasma cells. Although myeloma cells in case 12 showed a CD19–CD56+ phenotype when diagnosed at another hospital, the myeloma cells of this patient, who received allogeneic peripheral blood stem cell transplantation, showed a CD19–CD56– phenotype at our hospital. Thus, the phenotype of myeloma cells obtained from case 12 changed from CD19–CD56+ to CD19–CD56–.

The majority of normal plasma cells in 18 patients without MM after transplantation were CD19+CD56– but included a minor population of plasma cells with moderate CD56 expression. The major tendency was CD138 expression on myeloma plasma cells (Table 1). Myeloma plasma cells expressed higher levels of CD138 than normal plasma cells (Image 1B).

Fourteen BM samples from 7 patients were analyzed using a combination of CD38, CD45, and cytoplasmic immunoglobulin staining by 3-color flow cytometry

<table>
<thead>
<tr>
<th>Table 2</th>
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<tbody>
<tr>
<td>Phenotypic Analysis of Plasma Cells</td>
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</table>

<table>
<thead>
<tr>
<th>Case No.</th>
<th>M Protein</th>
<th>IEP</th>
<th>Plasma Cells in BM Smear (%)</th>
<th>Myeloma Cells: CD38, CD45 (%)</th>
<th>Immunophenotype (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IgA–λ</td>
<td>IgA–λ</td>
<td>50.0</td>
<td>35.8</td>
<td>50.0</td>
</tr>
<tr>
<td>2</td>
<td>IgA–λ</td>
<td>IgA–λ</td>
<td>0.2</td>
<td>0.196</td>
<td>0.7</td>
</tr>
<tr>
<td>6</td>
<td>IgA–λ</td>
<td>IgA–λ</td>
<td>0.2</td>
<td>0.020</td>
<td>0.7</td>
</tr>
<tr>
<td>7</td>
<td>IgG–κ</td>
<td>IgG–κ</td>
<td>6.4</td>
<td>2.238</td>
<td>36.0</td>
</tr>
<tr>
<td>11</td>
<td>IgA–κ</td>
<td>IgA–κ</td>
<td>2.4</td>
<td>0.000</td>
<td>2.8</td>
</tr>
<tr>
<td>12</td>
<td>IgG–λ</td>
<td>IgG–λ</td>
<td>1.8</td>
<td>0.864</td>
<td>0.3</td>
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<tr>
<td>13</td>
<td>IgG–κ</td>
<td>IgG–κ</td>
<td>2.0</td>
<td>0.882</td>
<td>7.0</td>
</tr>
</tbody>
</table>

BM, bone marrow; IEP, immunoelectrophoresis; IFE, immunofixation electrophoresis; PCR, polymerase chain reaction.

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significant difference ($P = .002$) between immunofixation-negative and immunofixation-positive cases.

Although we examined residual disease analysis in only 1 sample of peripheral blood obtained for autologous stem cell transplantation using a flow cytometric technique (case 6), the peripheral blood stem cell harvest in this case had detectable myeloma plasma cells (0.010% [51/495,712]) (data not shown).

As shown in Figure 2, of 5 cases after chemotherapy, 4 were associated with detectable paraprotein by immunofixation and a sequential decrease in myeloma plasma cell number as evaluated by immunoelectrophoresis. The remaining case with immunofixation-negative and PCR-negative in plateau phase had no detectable myeloma plasma cells. Of 5 cases after autologous transplantation, 2 with immunofixation-negative results in plateau phase had no detectable myeloma plasma cells. Case 16, characterized as immunofixation-negative, showed a sequential decrease in myeloma plasma cell number and finally had no detectable myeloma plasma cells. The remaining 2 cases with detectable paraprotein by immunofixation and immunoelectrophoresis showed no specific tendency in myeloma plasma cell numbers.

Of 3 cases after allogeneic transplantation, 1 with immunofixation-negative results in plateau phase had no detectable myeloma plasma cells. The remaining 2 with detectable paraprotein by immunofixation and immunoelectrophoresis showed no specific tendency in myeloma plasma cell numbers.

Discussion

In this study, we monitored the behavior of residual myeloma plasma cells in patients with MM after high-dose therapy and autologous or allogeneic transplantation using 3 different methods of a flow cytometric technique using 4-color staining against CD38/CD45/CD19/CD56, immunofixation, and PCR approaches. Flow cytometric analysis has not only been shown to be extremely effective in chronic lymphocytic leukemia using 4-color staining but also has been widely used as a method for residual disease analysis in MM using a 3-color staining approach. Plasma cell phenotypic aberrations by flow cytometry using a panel of monoclonal antibodies in quadruple combinations (CD38/CD56/CD19/CD45, CD138/CD28/CD33/CD56, and CD138/CD117/CD138/CD38) were identified at diagnosis and then used as patient-specific probes for follow-up analyses. 

![Figure 1](image1.png) Percentage of myeloma plasma cells in immunofixation-negative and immunofixation-positive cases with up to 5% myeloma plasma cells. $P = .002$. IFE, immunofixation electrophoresis.

![Figure 2](image2.png) Serial measurements of myeloma plasma cell numbers. A, After chemotherapy. B, After stem cell transplantation. Allo, allogeneic transplantation; Auto, autologous transplantation; IEP, immunoelectrophoresis; IFE, immunofixation electrophoresis; PCR, polymerase chain reaction.
We have demonstrated in this study that a relatively simple flow cytometric technique using 4-color staining against CD38/CD45/CD19/CD56 can distinguish neoplastic plasma cells from their normal counterparts on the basis of their CD19 and CD56 expression, even if both cell types are present within the same sample. Detectable myeloma plasma cells were found in 5 cases at diagnosis and 9 cases after treatment. Of 14 cases, 9 (64%) had CD19–CD56+ myeloma plasma cells, and 5 (36%) had CD19–CD56– myeloma plasma cells. The majority of normal plasma cells in 18 patients without MM after transplantation were CD19+CD56– but included a minor population of plasma cells having moderate CD56 expression. The major tendency was CD138 expression on myeloma plasma cells.

Myeloma plasma cells expressed higher levels of CD138 than normal plasma cells. The expression of CD138 is useful for making it possible to discriminate between the majority of plasma cells and other hematopoietic cells (particularly B-progenitor cells). Within the BM of patients with myeloma, CD138 is a specific marker for plasma cells and is not expressed by other hematopoietic cells or endothelial cells. Although myeloma cells in case 12 showed a CD19–CD56+ phenotype when diagnosed at another hospital, myeloma cells of this patient, who received allogeneic peripheral blood stem cell transplantation, showed a CD19–CD56– phenotype at our hospital. The phenotype of myeloma cells obtained from case 12 changed from CD19–CD56+ to CD19–CD56–. Pellat-Deceunynck et al reported that CD56– MM presented a leukemic phase more frequently than CD56+ MM. Sahara et al demonstrated that overall survival was significantly lower in CD56– MM than CD56+ MM. According to a previous report, 71% of myeloma plasma cells from 65 newly diagnosed patients with MM without evidence of extramedullary involvement showed high CD56 expression, and a mixture of CD56+ and CD56– myeloma plasma cell subsets were found in 5 of 65 cases. When compared with BM, CD56 expression was down-regulated in extramedullary myeloma plasma cells and peripheral blood. CD56 expression was down-regulated and CD44 expression was up-regulated in extramedullary myeloma cells.

Monoclonality was based on light chain restriction (κ/λ ratio, ≥ 5.0 or κ/λ ratio, ≤ 0.5). Of the 12 samples containing detectable myeloma plasma cells with immunoelectrophoresis-positive results, 11 with 0.196% or more myeloma plasma cells definitively showed plasma cell light chain restriction cells, whereas the 1 remaining sample with 0.020% myeloma plasma cells showed no detectable light chain restriction. These data show that analysis of 4-color flow cytometry against the combination CD38/CD45/CD19/CD56 is more sensitive than that of 3-color flow cytometry against the combination of CD38/CD45/cytoplasmic immunoglobulins. Although we examined residual disease analysis in only 1 peripheral blood sample obtained for autologous stem cell transplantation using a flow cytometric technique, the sample had detectable myeloma plasma cells (0.010%). The results of 37 BM samples with up to 5% myeloma plasma cells analyzed by flow cytometry were compared with the results of immunofixation performed on serum. Myeloma plasma cells were detected in all 20 immunofixation-positive cases and 3 of 17 immunofixation-negative cases. All 4 PCR-negative samples characterized as immunofixation-negative contained no detectable myeloma plasma cells. The detection of myeloma plasma cells resulted in a highly significant difference (P = .002) between immunofixation-negative and immunofixation-positive cases.

According to a previous report, 6 of 10 patients with immunofixation-positive results, 9 had positive minimal residual disease (MRD) by allelic-specific oligonucleotide real-time quantitative PCR (ASO-RQ-PCR), and 6 patients had positive MRD by flow cytometry. On the other hand, of 14 patients with immunofixation-negative results, 8 had positive MRD by ASO-RQ-PCR and 5 patients had positive MRD by flow cytometry. Although MRD evaluation by ASO-RQ-PCR was slightly more sensitive and specific than flow cytometry, it was applicable in a lower proportion of patients with MM and was more time-consuming, while both techniques provide similar prognostic information.

Furthermore, only 60% to 70% of patients have an amplifiable VDJ region. PCR assay methods can detect neoplastic plasma cells at the level of 1 in 10^5 leukocytes if no other B cells are present. If B cells are present, which is the case in most patient samples, the assay will identify a population that represents more than 2% of total amplifiable B cells, equating to a sensitivity of 1 in 10^3 to 1 in 10^4 total leukocytes. Analysis using consensus primer PCR is relatively insensitive and provides no additional information to that provided by immunofixation. All patients responding to treatment showed a serial decrease in the number of myeloma plasma cells in response to treatment, whereas patients later shown to have disease refractory to treatment showed no overall decrease in number. Case 16 with immunofixation-negative results showed a sequential decrease in myeloma plasma cell number and finally had no detectable myeloma plasma cells.

These data suggest that analysis of 4-color flow cytometry against the combination of CD38/CD45/CD19/CD56 is more sensitive than immunofixation. Flow cytometry can effectively detect low levels of myeloma plasma cells.

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