Elimination of Clonogenic Stem Cells From Human Multiple Myeloma Cell Lines by a Plasma Cell-Reactive Monoclonal Antibody and Complement

By Alex W. Tong, Jennifer C. Lee, Joseph W. Fay, and Marvin J. Stone

The monoclonal antibody (MoAb) MM4 reacts with human multiple myeloma (MM) cell lines and bone marrow from patients with plasma cell dyscrasias but not with normal peripheral blood or bone marrow cells. Treatment with MM4 and rabbit complement (C') was cytotoxic to the plasma cell-derived cell lines GM 1312, RPMI 8226, and ARH-77, as demonstrated by chromium release micromethodic toxicity and trypan blue exclusion assays. The same treatment eliminated >99% of clonogenic myeloma stem cell colony formation of these cell lines, with less than 20% inhibition of normal human bone marrow pleuripotent progenitor colony formation in vitro. As an experimental model to explore the efficacy of MM4 + C' in purging MM-involved bone marrow, normal marrow cells were mixed with RPMI 8226 or GM 1312 cells in the ratio of 90:10 or 50:50 (marrow:myeloma cells). Colony growth assays indicated that MM4 + C' eliminated at least 2 logs of clonogenic myeloma stem cells in both 90:10 and 50:50 preparations, while sparing the majority of normal marrow progenitors (inhibition of CFU-C:10% to 13%; BFU-E:0%).

The selectivity of MM4-mediated cytotoxicity may be useful for eliminating myeloma clonogenic stem cells from bone marrow of patients with multiple myeloma.

MUltiple myeloma (MM) represents a malignant plasma cell dyscrasia (PCD) with a wide spectrum of clinical manifestations. In 1986 it was estimated that approximately 10,000 new patients were diagnosed with this disorder in the United States. With the use of alkylating agent chemotherapy regimens, objective remission is possible for 30% to 60% of the myeloma patient population. Despite improvement in quality and duration of remission, the median survival for MM patients is limited to 3 to 4 years from time of diagnosis and has not improved substantially during the past 20 years. MM is in the advanced stages of its natural history at time of diagnosis, and patients responding to therapy rarely have more than a 1- to 2-log reduction (90% to 99%) of their neoplastic mass. A large residual tumor burden, or the emergence of a drug resistant subclone, accounts for the high incidence of tumor relapse. Patients with MM usually die of progressive disease, infections due to immunosuppression commonly associated with MM, or renal failure.

Adjuvant bone marrow (BM) transplantation can provide the option of extending the dose range of cytotoxic chemotherapeutic agents to improve patient chemoresponsiveness and to prolong survival. This approach has proven effective for some patients with primary hematopoietic malignancies, including refractory non-Hodgkin’s lymphomas and acute and chronic granulocytic leukemias. Marrow transplantation studies on MM patients have been limited. In a clinical trial of five MM patients, transplantation of syngeneic marrow accompanying massive chemotherapy extended survival. However, the likelihood of success using allogeneic marrow transplantation is limited by age and/or performance considerations of the MM patient population. Whereas appropriate monoclonal antibodies (MoAbs) can serve as tumor-purging reagents for preparing autologous BM for transplantation in other hematologic malignancies, this approach has not been extensively investigated in MM. The majority of plasma cell-reactive MoAbs described also react with granulocytes or lymphocytes or do not bind the majority of patient myeloma cells.

In a previous report we described a myeloma cell-reactive MoAb, MM4, generated in this laboratory. MM4 reacted with a 40-kd antigenic determinant that was detected on seven of seven human plasma cell-derived lines and on the majority of plasma cells in all of 18 BM biopsies from patients with various plasma cell dyscrasias (MM, Waldenström’s macroglobulinemia, or asymptomatic plasma cell dyscrasia). By contrast, MM4 did not react with BM biopsies or peripheral blood mononuclear (PBM) cells from patients with lymphocytic or granulocytic leukemias. The MM4-reactive antigen was not expressed on normal PBM cells, granulocytes, or erythrocytes.

In this study we examined the complement-mediated cytotoxicity of MM4 with plasma cell-derived cell lines, normal peripheral blood, and bone marrow cells. In an experimental model of normal bone marrow mixed with cells from different human MM-derived cell lines, MM4 + C’ eliminated myeloma clonogenic stem cells while preserving most normal BM progenitor cells. These observations suggest that this plasma cell-reactive MoAb may be of value for selectively eliminating myeloma cells from MM marrow ex vivo.

MATERIALS AND METHODS

Human multiple myeloma-derived cell lines. The plasma cell line GM 1312 was obtained from the NIGMS Human Genetic Mutant Cell Repository, Institute of Medical Research, Camden, NJ. The multiple myeloma-derived RPMI 8226, plasma cell leuke-

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Myeloma-derived ARH-77, and plasmacytoma-derived HS-Sultan lines were obtained from the American Type Culture Collection, Rockville, MD, as described previously.27 The mouse myeloma cell line SP2/0-Ag14 was obtained from Dr Denis Burger, Portland, OR.

Patient blood and bone marrow specimens. Human PBM cells and BM samples from healthy controls or patients with hematologic malignancies were procured according to the protocol approved by the Institutional Review Board for Human Protection, Baylor University Medical Center (BUMC).

Patients with hematologic malignancies were diagnosed and classified independently by the clinical staff, Department of Oncology, Charles A. Sammons Cancer Center, BUMC. Sections of paraffin-embedded BM biopsies from these patients were obtained from the Department of Pathology, BUMC, after routine histopathologic evaluation of the specimens.

Preparation of BM + MM cell mixtures. Normal marrow mononuclear cells were derived from Hypaque Ficoll centrifugation (400 g, 20 minutes) of marrow aspirates and resuspended in RPMI 1640 at 10⁶/mL. For the preparation of BM + MM cell mixtures, these were mixed with appropriate volumes of either RPMI 8226 or GM 1312 cells (10⁶/mL) to achieve normal marrow:myeloma cell ratios of 90:10 or 50:50.

Production of MoAbs. MM4 was generated by an alternate immunization protocol, as described previously.27-29 Briefly, BALB/c mice were immunized alternately with 1 x 10⁶ live cells from the RPMI 8226 and GM 1312 cell lines. Somatic hybridization of the sensitized spleenocytes with SP2/0-Ag14 myeloma cells (1:1, spleenocyte:myeloma cells) was performed three days after the last injection, utilizing 50% polyethylene glycol and 5% dimethyl sulfoxide according to the method of Tong et al.30-32 and Oi and Herzenberg.33 Syngeneic thymocytes served as "filler cells" (10⁵ per well) during propagation. Antibody reactivity of confluent hybridomas was characterized by ELISA against MM cell lines and peripheral blood cells of MM patients.27'28

Limiting dilution analysis. Limiting dilution analysis of clonogenic units was determined using a Spearman estimate.32-38 A series of ten fivefold dilutions were prepared from MM4 + C-treated or untreated normal bone marrow preparations containing 10%
myeloma cells from the cell lines RPMI 8226, GM 1312, or ARH-77 that initially contained 5 x 10^5 tumor cells/mL. Five replicates (100 μL) of each dilution were plated in 6-well flat-bottomed tissue culture plates (Falcon) onto methylcellulose under the culture conditions described above. The mean value for each treatment group was estimated by:

\[ M = X_0 + d/2 - d/n \sum_{i=0}^{k} r_i; \]

where \( X_0 = \) ln initial dose - ln 10; \( d = \) ln dilution factor = ln 5; \( n = \) number of replicates per dilution = 5; \( k = \) number of fivefold dilutions = 9; and \( r_i = \) the number of wells with observed growth at the \( i \)th dilution. The estimated number of clonogenic units per milliliter was calculated as \( \Theta = \exp (-0.5772 - M) \), with variance approximated by \( d \ln 2/n \).

RESULTS

**MM4-mediated cytotoxicity.** Cytotoxicity of MM4 and C' against cells from established plasma cell dyscrasia (PCD)-derived cell lines was determined by short-term chromium release assays. Exposure to the IgG1 MoAb MM4 at 0.015 to 1 mg per 10^5 cells followed by rabbit C' at the pretitered final dilution of 1:16 was cytolytic to 25% to 95% of cells from the PCD cell lines tested (Fig 1). The LD50s of MM4 + C'-mediated cytotoxicity were 0.015 mg, 0.047 mg, and 0.045 mg for GM 1312, RPMI 8226, and ARH-77 cells respectively, with a dose-response pattern of the ARH-77 line almost identical to that of RPMI 8226 (Fig 1). Toxicity to normal PBM cells (20% ± 7.5%) was evident after incubation with MM4 at 1 mg. Subsequent determinations thus were conducted at the optimal MoAb concentration of 0.5 mg/10^5 cells.

Similar to the observations of others,16,18,19 two cycles of C' incubation (1:16 final dilution) improved cytosis of MM4-bound tumor cells to 94.5%, 72.3%, and 80.5% for GM 1312, RPMI 8226, and ARH-77 lines respectively (Table 1). There was no appreciable increase in background toxicity by C' alone, with a mean cytolytic of 18.75% ± 5.25% with the three cell lines tested. Under the same experimental conditions, evaluations by trypan blue exclusion demonstrated that <1% of GM 1312 cells were viable (Table 1) after MM4 + C' treatment, despite a lower level of chromium release (94.5%). The same treatment yielded less than 6% viable RPMI 8226 cells, according to trypan blue exclusion studies. Higher background toxicity and a corresponding increase in normal BM and PBM cell lysis were observed if complement treatments were extended to three or more cycles (data not shown) or if a higher concentration of complement was used (2 x 1:8 or 1:16, Table 1). Such variations also did not result in significant improvement of cell kill, as determined by trypan blue exclusion (Table 1). Re-exposure to MM4 after complement treatment did not enhance myeloma-specific cytosis.

**Depletion of clonogenic stem cells from MM cell lines.** Tumor repopulation and/or metastatic growth have been postulated to be mediated by a self-renewing tumor stem cell subset. Chemoresponsiveness of clonogenic stem cell cultures of patients in vitro has been correlated with subsequent clinical response.39,41 Previously, MM4 alone has been found to inhibit clonogenic colony formation of RPMI 8226 cells, with an LD50 of 0.01 to 0.1 mg/10^5 cells.27 We carried out clonogenic stem cell assays to establish the effect of MM4 + C' on clonogenic stem cells from PCD cell lines. MM4 + C' completely abrogated myeloma clonogenic stem cell colony formation of GM 1312, RPMI 8226, and ARH-77 cell lines under optimized conditions (Table 1). The inhibition of normal marrow stem cell colony formation (CFU-C) under the same conditions was limited to 14.6% and 7.5%, after exposure to complement at 1:8 and 1:16 dilutions respectively.

**Elimination of myeloma cells from bone marrow.** As an experimental model to test the efficacy of MM4 + C' in eliminating myeloma cells from involved patient marrow, cells from the RPMI 8226 or GM 1312 lines were mixed with normal bone marrow mononuclear cells in final proportions of 50:50 and 90:10 (normal marrow:myeloma cells). These ratios were designed to approximate the content of myeloma cells in BM of patients prior to treatment and in remission.

As shown in Table 2 with GM 1312 cells, the efficacy of MM4 + C' in eliminating myeloma clonogenic stem cells from the tumor cell line was not affected by the presence of normal BM cells (Table 2). However, the overall capacity of this treatment in lysing tumor cells was diminished based on chromium release data. A mean cytolytic value of 83.8% ± 18.7% for GM 1312, RPMI 8226, and ARH-77 lines respectively (Table 1). There was no appreciable increase in background toxicity by C' alone, with a mean cytolytic of 18.75% ± 5.25% with the three cell lines tested. Under the same experimental conditions, evaluations by trypan blue exclusion demonstrated that <1% of GM 1312 cells were viable (Table 1) after MM4 + C' treatment, despite a lower level of chromium release (94.5%). The same treatment yielded less than 6% viable RPMI 8226 cells, according to trypan blue exclusion studies. Higher background toxicity and a corresponding increase in normal BM and PBM cell lysis were observed if complement treatments were extended to three or more cycles (data not shown) or if a higher concentration of complement was used (2 x 1:8 or 1:16, Table 1). Such variations also did not result in significant improvement of cell kill, as determined by trypan blue exclusion (Table 1). Re-exposure to MM4 after complement treatment did not enhance myeloma-specific cytosis.

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Table 1. Effect of MM4 + Complement Treatment on Multiple Myeloma Cell Lines and Normal Bone Marrow

<table>
<thead>
<tr>
<th>Target</th>
<th>Cytolysis*</th>
<th>% Nonviable Cells†</th>
<th>% Inhibition Of Colony Formation‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:8</td>
<td>1:16$</td>
<td>1:8</td>
</tr>
<tr>
<td>MM cell lines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM 1312</td>
<td>96.1 ± 3.1</td>
<td>94.5 ± 4.6</td>
<td>97.1 ± 3.9</td>
</tr>
<tr>
<td>RPMI 8226</td>
<td>80.8 ± 3.3</td>
<td>72.3 ± 2.5</td>
<td>93.5 ± 5.6</td>
</tr>
<tr>
<td>ARH-77</td>
<td>88.0 ± 4.3</td>
<td>80.5 ± 2.2</td>
<td>NT</td>
</tr>
<tr>
<td>Normal bone marrow</td>
<td>34.3 ± 8.7</td>
<td>20.1 ± 8.7</td>
<td>NT</td>
</tr>
</tbody>
</table>

*Complement treatment was determined by % specific chromium release microcytotoxicity assay after incubation with MM4 (0.5 mg/10^8 cells; 4°C, 60 minutes) and rabbit complement (23°C, 30 minutes x 2) at the indicated dilution. Value represents mean ± SD (n = 5).
†Determined by trypan blue exclusion (see Materials and Methods).
‡Determined by stem cell colony-growth assay for treated myeloma cell lines and CFU-C colony formation for normal bone marrow. Myeloma stem cell colonies were scored on day 7, and CFU-C on day 10. Value represents Mean ± SD (n = 4).
§Final complement dilution.
||NT, not tested.

Table 2. Effect of MM4 + Complement Treatment on Cell Preparations Containing Normal Bone Marrow and Myeloma Cells

<table>
<thead>
<tr>
<th>BM:GM 1312*</th>
<th>MM4 Added (mg)</th>
<th>% Colony Inhibition†</th>
<th>% Specific 51Cr Release‡</th>
<th>% Inhibition of BM Stem Cells§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CFU-C</td>
</tr>
<tr>
<td>90:10</td>
<td>0.5</td>
<td>99.5 ± 0.5</td>
<td>83.8 ± 9</td>
<td>13.5 ± 10</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>95.1 ± 4</td>
<td>78.4 ± 11</td>
<td>10.2 ± 10</td>
</tr>
<tr>
<td>50:50</td>
<td>0.5</td>
<td>100 ± 0</td>
<td>59.5 ± 14</td>
<td>13.3 ± 13</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>92.4 ± 4</td>
<td>51.5 ± 5</td>
<td>7.1 ± 7</td>
</tr>
</tbody>
</table>

*BM:GM 1312 cells were mixed with normal bone marrow mononuclear cells in the indicated ratios.
†Determined by stem cell colony-growth assay on day 7 and differentiated from marrow colonies by their distinctive colony morphology and irregular growth pattern. Value represents mean ± SD (n = 4).
‡Determined by short-term microcytotoxicity assay (see Materials and Methods). Mean ± SD (n = 3).
§CFU-C colonies were scored on day 10 and identified by their lack of color and relatively diffuse pattern. BFU-E were recognized by their red color and tightly packed colonies on day 14. Value represents mean ± SD (n = 4).
Fig 2. Photomicrographs of stem cell colonies of MM cell lines and normal bone marrow. In cell preparations with normal marrow and MM cells, MM colonies were distinguished by their larger cell size, faster growth rate, and distinctive colony morphology. GM 1312 colonies (insert A) were characterized by their tightly packed spherical conformation, whereas RPMI 8226 colonies (insert B) were comprised of tightly aligned tumor cells. By comparison, CFU-C colonies had a diffuse growth pattern, such as the type IV colony (see reference 37) shown in insert C. BFU-E colonies (insert D) were characterized by their late emergence, red color, and small, tightly packed colony morphology. Original magnification x 100.

to studies by Matsuoka et al and others, the IgG, lambda RPMI 8226 cell line is comprised mainly of immature plasma cells and reacts with both PCA-1 and OKT10. The IgG, kappa GM 1312 cell line is morphologically less well differentiated, is unreactive with OKT10, and only weakly reactive with PCA-1. The IgG kappa-producing plasma cell leukemia cell line ARH-77 displays a variety of morphological entities ranging from small lymphocytes to classic plasma cells and reacts with PCA-1 but not OKT10. The heavy and light chain isotype of these cell lines as well as their immunologic phenotype were confirmed in this laboratory prior to and after culture under clonogenic
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Stem cell colony forming assay conditions. In this study it was evident that greater than 90% of cells from all three cell lines bound MM4, the majority of which were susceptible to complement-mediated cytolysis. The LD50s of 0.015 to 0.047 mg of MM4 per 10^6 cells were consistent with MoAb concentrations employed for cytolysis of other human tumors.\(^ {17,19}\) Parallel trypan blue exclusion studies indicated that MM4 + C' was effective in killing >94% of total cells from the PCD cell lines tested.

More significantly, MM4 + C' consistently abrogated clonogenic stem cell colony growth of the PCD cell lines tested, indicating that the clonogenic stem cell subset expressed the MM4-reactive antigen. As shown in this and our prior study,\(^ {27}\) MM4 alone was effective in inhibiting clonogenic stem cell colony formation of MM cell lines by an as yet undefined mechanism. The inhibitory effect was amplified by subsequent incubation with C', and efficacy of clonogenic stem cell elimination was maintained in our experimental model of normal bone marrow cell preparations mixed with myeloma cells. MM4 + C' eliminated 2 logs or more of clonogenic myeloma stem cells both in 90:10 and 50:50 preparations from RPMI 8226, GM 1312, or ARH-77 cell lines. The complement-inhibitory effect of BM cells, also documented previously by other investigators,\(^ {17,19}\) was evident in chromium release data but apparently did not affect the capacity of MM4 + C' in deleting the clonogenic myeloma stem cell subset. Alternatively, this discrepancy may be related to assay artifacts, such as the resorption of released chromium from the lysed MM cells by normal bone marrow bystanders.

In an earlier report\(^ {27}\) we found that approximately 70% of the total plasma cells in BM biopsies of MM patients express the MM4-reactive antigen. Our studies on established MM cell lines strongly suggest that the clonogenic stem cell subset from myeloma patients also expresses MM4. Whereas heterogeneity of antigen expression precludes the possibility of completely eradicating the entire myeloma/plasma cell population by MM4-mediated cytolysis, the same treatment can selectively eliminate the clonogenic stem cell subset. Several lines of evidence support the thesis that responsiveness of clonogenic stem cell cultures to cytotoxic or other agents correlate with in vivo therapeutic response.\(^ {39,47}\) In patients with multiple myeloma, Karp et al.\(^ {48}\) and others\(^ {3,49}\) have shown that clonogenic colony-forming cells from MM marrow are closely related to kinetically active myeloma cells that proliferate in vivo. Elimination or major reduction of this stem cell subset may limit expansion of the myeloma cell clone with potentially advantageous therapeutic consequences.

Recent studies with leukemia and lymphoma cells have indicated success in purging malignant tumor cells from involved BM with the appropriate MoAbs, thereby providing the option for autologous marrow reconstitution following marrow-ablative chemoradiotherapy.\(^ {12-14,16}\) At present this therapeutic alternative is not available for patients with MM, despite syngeneic marrow transplantation studies suggesting that dose escalation may overcome apparent drug resistance in patients with refractory disease.\(^ {15}\) With the use of alkylation agent-predisone regimes, it is possible to achieve myeloma cell reduction to 10% or less of BM content in some MM patients.\(^ {3,49}\) Our experimental model suggests that it may be possible to further reduce the BM myeloma stem cell content by an additional 2 logs or more by incubating with MM4 + C', thereby potentially achieving a total reduction of 3 logs or more of myeloma stem cells. Under the same experimental conditions, toxicity to CFU-C

**Table 3. Efficacy of MM4 + Complement in Eliminating Multiple Myeloma Clonogenic Stem Cell Colony Formation**

<table>
<thead>
<tr>
<th>Cell Lines*</th>
<th>Prior Treatment (C)</th>
<th>After Treatment (C)</th>
<th>% Reduction†</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI 8226</td>
<td>7.83 ± 3.68 x 10³</td>
<td>45.47 ± 21.37</td>
<td>99.42</td>
</tr>
<tr>
<td>GM 1312</td>
<td>1.639 ± 0.770 x 10³</td>
<td>18.19 ± 8.549</td>
<td>98.89</td>
</tr>
<tr>
<td>ARH-77</td>
<td>7.831 ± 3.681 x 10³</td>
<td>2.512 ± 1.180</td>
<td>99.97</td>
</tr>
<tr>
<td>HS-Sultan</td>
<td>0.982 ± 0.452 x 10³</td>
<td>1.915 ± 0.900</td>
<td>99.81</td>
</tr>
</tbody>
</table>

*Mixed with normal bone marrow mononuclear cells in the ratio of 90:10 (marrow:myeloma cells).
†Determined by limiting dilution and the Spearman estimate (see "Materials and Methods").
‡% Reduction = (C₀ - C)/C₀ x 100%.
concentration, whereas reactivity with normal PBM cells attributable to background toxicity from C' alone. Alterna-

tively, we cannot exclude the possibility that the MM4-reactive antigen may be expressed on some normal BM progenitor cell colonies following purging procedures that involve MoAbs unreactive with normal plasma cells, which are present at low levels in normal marrow. Other studies also have demonstrated highly variable BM recovery (50% or less) in terms of cell number or progenitor cell colonies following purging procedures that involve MoAbs unreactive with normal BM. However, patient marrow reconstitution does not appear to be affected by such losses. Our approach of

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