Autologous Bone Marrow Transplantation in Acute Myelogenous Leukemia: In Vitro Treatment With Myeloid Cell-Specific Monoclonal Antibodies

By Edward D. Ball, Letha E. Mills, Christopher T. Coughlin, J. Robert Beck, and Gibbons G. Cornwall III

Second or third chemotherapy-induced remissions in acute myelogenous leukemia (AML) are limited by early relapse of the leukemia. We developed monoclonal antibodies (MoAbs) that are cytotoxic to myeloid leukemia cells to treat bone marrow from these patients ex vivo for autologous transplantation. In this pilot study, bone marrow was harvested from ten patients with AML in remission, treated with one or two complement-fixing MoAbs, PM-81 and AML-2-23, which react with myeloid differentiation antigens, incubated with rabbit complement, and cryopreserved. These MoAbs were chosen because they have broad reactivity with AML cells but not with pluripotent progenitor cells. At the time of transplant, 6 patients were in second complete remission, 1 each was in third complete or partial remission, and 2 were in early first relapse. The patients were treated with cyclophosphamide (60 mg/kg a day for 2 days) and total body irradiation (200 cGy twice a day for 3 days) and given infusions of MoAb-treated bone marrow. Full bone marrow reconstitution was observed in eight patients; two patients did not recover platelets.

Seven of the ten patients are surviving and disease-free at 21.0, 15.0, 13.0, 10.0, 6.0, 3.0, and 2.0 months posttransplant. Treating bone marrow with MoAbs to myeloid differentiation antigens does not interfere with pluripotent stem cell engraftment. Longer follow-up and a controlled study are necessary to prove that the apparent efficacy of this therapeutic approach in some patients is attributable to MoAb-mediated killing of leukemia cells.

MATERIALS AND METHODS

Monoclonal antibody purification. MoAbs PM-81 and AML-2-23 were purified from ascites as previously described. All MoAb preparations were tested for sterility by culture in tryptic soy broth and for endotoxin by the Limulus amoebocyte lysate assay.

Surface marker analysis of leukemia cells. Blast cells obtained from bone marrow at the time of diagnosis or first relapse were separated by Ficoll-Hypaque gradient centrifugation as described and incubated with purified MoAb (100 µl at 20 µg/mL) for 60 minutes at 4°C in the presence of human IgG (10−3 mol/L) to block Fc receptor binding. After cells were washed with phosphate-buffered saline (PBS) (pH 7.4) containing bovine serum albumin (BSA) (0.1%) and sodium azide (AZ) (0.5%), a fluoresceinisothiocyanate-labeled goat anti-mouse Ig F(ab')2 antibody (Boehringer-Mannheim, Indianapolis) was added for a further incubation of 30 minutes at 4°C. After being washed with PBS/BSA/AZ, the cells were fixed with 1% paraformaldehyde and analyzed on the Ortho (Westwood, MA) Cytofluorograf system 50H with the 2150 computer system.

Complement-mediated cytotoxicity. Cytotoxicity of MoAbs for leukemia cells was assessed by incubation of cells with MoAbs (as above) followed by the addition of neonatal rabbit serum (Pel Freez, Rogers, AR) to achieve a final dilution of 1:6. After being incubated for 21.0, 15.0, 13.0, 10.0, 6.0, 3.0, and 2.0 months posttransplant...
for 1 hour at 22°C with continuous mixing, the cells were washed and their viability was assessed using acridine orange and ethidium bromide.16

Processing and treating of bone marrow cells. An average of 6.3 x 10^6 (SE 0.8 x 10^6; range 3.5 to 11.5 x 10^6) nucleated bone marrow cells per kilogram of body weight were removed through multiple punctures of the anterior and posterior iliac crests of patients under general anesthesia and collected in Medium-199 (GIBCO, Grand Island, NY) containing preservative-free heparin (Sigma Chemical Co, St Louis). Mononuclear cells were separated by centrifugation on a Haemonetics (Braintree, MA) Model 30 cell processor17 with a mean recovery of 1.5 x 10^6 (SE = 0.47 x 10^6, range 0.4 to 7.6 x 10^6) cells per kilogram of body weight. The cells were incubated on a platform shaker with purified MoAbs PM-81 (50 μg/mL) alone (patients 4 through 6) or with MoAbs PM-81 and AML-2-23 (both at 50 μg/mL) (patients 1 through 3 and 7 through 10) for 15 minutes, followed by the addition of neonatal rabbit serum to achieve a final dilution of 1:6.18 Deoxyribonuclease Type I (10 U/mL) (Sigma) was added to the cell suspension to decrease the tendency of cells to aggregate following lysis. After 60 minutes at room temperature, the cells were sedimented at 500 g, and the supernatant was removed. This process was repeated, and the cells were assessed for viability and adjusted to 8 x 10^6 cells/mL with Medium-199. The mean recovery of cells after MoAb treatment was 2.6 x 10^6 (SE = 0.34 x 10^6, range 1.8 to 5.4 x 10^6) cells per kilogram of body weight. A solution of Medium-199 containing 20% dimethylsulfoxide (Rimso-100, Research Industries Corporation, Salt Lake City) and 10% irradiated (1,500 rad) autologous plasma was added in equal volume to the cell suspension, which was then transferred to liquid nitrogen for storage.

Effect of MoAb + complement treatment on granulocyte/monocyte progenitor cells. The ability of the patients' bone marrow cells before and after MoAb therapy to form colonies in methylcellulose cultures in the presence of colony-stimulating factor was measured as previously described.8 After 14 days, CFU-GM were counted on an inverted microscope.

Ablative therapy and autologous bone marrow transplantation (ABMT). Cytoablation therapy consisted of cyclophosphamide (60 mg/kg body weight on days 5 and 4), followed by total body irradiation (TBI) (1,200 cGy total in twice-daily doses 6 hours apart of 200 cGy on days −3, −2, and −1) delivered with a Cobalt-60 source by opposed lateral fields without lung shielding.19 On day 0, frozen bone marrow was quickly thawed at 38°C and administered through a central venous line. All patients received intrathecal methotrexate (12 mg) on day −3.

Patient characteristics. Patients ranged in age from 16 to 44 years and included six female and four male patients (Table 1). Five patients had their marrow harvested in first CR, four had their marrow harvested in second CR, and one had marrow harvested in third CR. Transplantation was performed after a second CR was achieved in three patients (nos. 4, 8, and 10) previously harvested in first CR and at early relapse (9% myeloblasts in the bone marrow in patient 6 and a breast chloroma in patient 7) in two other patients harvested in first CR. Three patients (nos. 1, 2, and 9) harvested in second CR were also transplanted in second CR. One patient (no. 5) whose marrow was harvested in second CR relapsed before transplant could be performed. After an attempt was made to reinduce remission with homoharringtonine, his bone marrow biopsy was hypocellular and contained a small focus of blasts at the time of ABMT. The patient (no. 3) whose marrow was harvested in third remission was transplanted in third remission. Informed consent was obtained for all aspects of the bone marrow transplant following guidelines approved by the Committee for the Protection of Human Subjects of the Dartmouth-Hitchcock Medical Center.

All patients had received cytosine arabinoside (ARA-C) and daunorubicin (DNR) as their initial remission induction chemotherapy. Reinduction therapy for patients 1 through 3 consisted of ARA-C (patient 1 received 3 g/m² every 12 hours for 2 days; patients 2 and 3 received 100 mg/m² a day for 7 days). Patients 4, 8, and 10 achieved CR with mitoxantrone. Patient 5 was treated with homoharringtonine at second relapse. Patient 6 was transplanted at the first sign of bone marrow relapse after 20 months of remission following ARA-C/DNR induction and postremission intensification. Patient 7 was transplanted at the time a breast chloroma was noted. The chloroma disappeared following treatment with electron-beam radiotherapy (1,000 cGy), but no systemic therapy was used other than the ablative therapy described above. Patient 9 was reinduced with ARA-C (100 mg/m²/day x 7 days)/DNR (30 mg/m²/day x 3 days)/Vincristine (1 mg/m² x 1).

RESULTS

Surface marker analysis. Cytofluorographic analysis of the patients’ leukemia cells revealed that 21% to 91% of cells were positive for the PM-81 marker (Table 2). Complement-mediated cytotoxicity of 52–100% of cells from individual patients was achieved with this MoAb. Cells (FAB M4) from three patients (nos. 1, 8, and 10) also reacted significantly with MoAb AML-2-23 (Table 2). Cells from two patients (nos. 2 and 9) were not available for analysis since the patients were first referred to us in second remission. Our experience is that blasts from >93% of patients with FAB M4 AML react with both MoAbs PM-81 and AML-2-23.10,11

Engraftment. The first neutrophils appeared by posttransplant day 9 in all patients. The mean time to reach a neutrophil count of >500 cells/μL was 27 days (±5.4, range 12 to 60 days). Reticulocytes appeared in the blood on approximately day 20, and the hemoglobin levels were

### Table 1. Nos. of CFU-GM Before and After MoAb Treatment

<table>
<thead>
<tr>
<th>Patient</th>
<th>CFU-GM/10⁶ Cells Before</th>
<th>CFU-GM/10⁶ Cells After</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total CFU-GM*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7.98 x 10⁴</td>
<td>2.03 x 10⁴</td>
<td>75%</td>
</tr>
<tr>
<td>2</td>
<td>1.61 x 10⁴</td>
<td>0.68 x 10⁴</td>
<td>58%</td>
</tr>
<tr>
<td>3</td>
<td>1.04 x 10⁴</td>
<td>1.08 x 10⁴</td>
<td>0%</td>
</tr>
<tr>
<td>4</td>
<td>10.6 x 10⁴</td>
<td>0.56 x 10⁴</td>
<td>-95%</td>
</tr>
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<td>5</td>
<td>2.64 x 10⁴</td>
<td>1.62 x 10⁴</td>
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</tr>
<tr>
<td>6</td>
<td>2.64 x 10⁴</td>
<td>1.08 x 10⁴</td>
<td>-59%</td>
</tr>
<tr>
<td>7</td>
<td>2.24 x 10⁴</td>
<td>1.40 x 10⁴</td>
<td>-37%</td>
</tr>
<tr>
<td>8</td>
<td>0.72 x 10⁴</td>
<td>0.77 x 10⁴</td>
<td>0%</td>
</tr>
<tr>
<td>9</td>
<td>1.6 x 10⁴</td>
<td>0.98 x 10⁴</td>
<td>-39%</td>
</tr>
<tr>
<td>10</td>
<td>1.6 x 10⁴</td>
<td>2.4 x 10⁴</td>
<td>+50%</td>
</tr>
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</table>

Average: 3.26 ± 1.36 (±0.25 x 10⁴)
Table 2. Phenotype of Patients' Leukemia Cells as Determined by Flow Cytometry and Complement-Dependent Cytotoxicity

<table>
<thead>
<tr>
<th>Patient</th>
<th>FAB</th>
<th>PM-8</th>
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<th>AML-2-23</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>IF</td>
<td>C'</td>
<td>IF</td>
<td>C'</td>
</tr>
<tr>
<td>1</td>
<td>M2</td>
<td>77</td>
<td>81</td>
<td>32</td>
<td>64</td>
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<td>M4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>M2</td>
<td>83</td>
<td>90</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>M1</td>
<td>91</td>
<td>100</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>M2</td>
<td>67</td>
<td>75</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>M1</td>
<td>40</td>
<td>55</td>
<td>2</td>
<td>16</td>
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<tr>
<td>7</td>
<td>M4</td>
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<td>92</td>
<td>70</td>
<td>85</td>
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<tr>
<td>9</td>
<td>M2</td>
<td>68</td>
<td>85</td>
<td>44</td>
<td>74</td>
</tr>
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</table>

FAB: French-American-British classification; IF, immunofluorescence; ND: not done.

Blasts from patients with AML were examined by indirect immunofluorescence and flow cytometry for the expression of the PM-81 and AML-2-23-defined antigens. Background staining from irrelevant isotype control monoclonal antibodies (MoAbs) has been subtracted from the percentage values shown; 10,000 cells were examined in each analysis. Cells were incubated with MoAb and rabbit complement (C') for 60 minutes, and percentage of cytotoxicity was estimated by dye exclusion (acridine orange/ethidium bromide).

sustained at >10 g/dL without transfusions of RBCs by day 39 (±5.9, range 32 to 62 days) on average. Recovery of platelets was generally slow. Although eight of the ten patients no longer required platelet transfusions by day 45 (platelet count ≥ 20,000/µL), platelet counts of >50,000/µL were not reached until a mean time of 60 ± 20 days (range 45 to 150 days). A normal platelet count was not achieved in patient 1 until at least day 280 posttransplant (Fig 1). We observed a similar time course of platelet recovery in patients 3 through 8. Two patients (nos. 2 and 7) never achieved sustained platelet counts >20,000/µL. Bone marrow biopsy in these patients showed decreased megakaryocytes and slight hypocellularity. One ultimately died of a massive subarachnoid hemorrhage (no. 2), and one (no. 7) died of respiratory failure secondary to infection.

Toxicity. Patients suffered the expected side effects of radiotherapy and cyclophosphamide, including nausea, vomiting and mucositis. All patients required treatment with multiple antibiotics, including amphotericin B for proven or suspected candidiasis. Patients 2 and 7 had persistent thrombocytopenia which contributed to their deaths. There was no evidence of leukemia pre- or postmortem in either patient.

Effect of MoAb + C' treatment on CFU-GM. Bone marrow cells before and after MoAb + C' treatment were cultured for 14 days in methylcellulose and examined for CFU-GM content. The number and concentration of CFU-GM varied from patient to patient before MoAb treatment, reflecting at least a variation in the assay from experiment to experiment and possibly in the true number of CFU-GM harvested (Table 1). The degree of variation after MoAb + C' treatment was less significant. Overall, the effect of MoAb + C' was to decrease CFU-GM absolute numbers by an average of 45%.

Outcome. Seven patients are surviving disease-free following ABMT (Table 3). The longest survivors are 630+; 450 +, 380 +, and 300 + days posttransplant. These patients are enjoying a high quality of life and are hematologically normal or nearly so. One patient who was in partial remission died following relapse of leukemia, and two died while still in leukemic remission 230 and 180 days posttransplant. In summary, six of seven patients transplanted in second or third CR are surviving and disease-free at 60+, 90+, 180+, 390+, 450+, and 630+ days.

DISCUSSION

We have described our initial experience in a phase I study of the use of antilymphoid MoAbs to treat remission bone marrow ex vivo prior to its autotransplantation in patients with AML. This is the first report of the use of MoAbs directed to myeloid-differentiation antigens for this purpose and is significant for two reasons. First, although engraftment of normal myeloid cells was delayed, all patients ultimately recovered normal granulocyte levels despite significant reductions in the absolute numbers of CFU-GM after MoAb treatment (45% on average). The second significant albeit preliminary observation is that long periods of leukemia-free survival have resulted from this program in patients who historically would have developed early relapses. Although the number of patients we have treated is small, the patients’ remission durations are promising. The three patients transplanted in second CR with the longest follow-up are surviving at 180+, 390+, and 630+ days; another patient in second CR (no. 2) who died of thrombocytopenia was in leukemic remission at 230 days posttransplant. The expected median survival of patients in second remission following high-dose ARA-C reinduction is ≤5 months.20 All our patients transplanted in second CR who have been followed long enough have exceeded 5 months of disease-free survival. The patient transplanted in third CR is surviving and disease-free 450+ days posttransplant. This duration of remission is rare in third remission. Although these initial results are encouraging, definitive conclusions

![Fig 1](image-url)
regarding the curative potential of this program require more time, more patients, and a larger clinical trial.

It will be important to determine whether these initial promising results are related to the in vitro MoAb treatment. For example, ABMT in AML without purging of leukemia cells has been reported.\textsuperscript{21-25} When performed in relapse or second remission, ABMT has not produced long remissions\textsuperscript{21,22}; this method has been more successful in first remission, however.\textsuperscript{23-25} Eleven of 29 (38\%) patients in first CR have achieved long disease-free survival (\textgreek{\textalpha} 2 years) in these studies, with an overall median survival of 63 weeks. Fourteen of 29 patients in these trials relapsed within 24 months, however. In addition to immunologic methods, cytotoxic drugs are potentially useful for purging marrow of residual leukemia blasts. Kaizer and colleagues reported several long-term survivors with AML transplanted with autologous marrow treated in vitro with the cyclophosphamide congener, 4-hydroperoxycyclophosphamide (4HC).\textsuperscript{26} Definitive clinical trials will be necessary to demonstrate the relative efficacy of these various therapeutic approaches.

This study addresses the fundamental scientific question of whether it is possible to eliminate the leukemic clone of cells from patients with AML using MoAbs directed to antigens that normally appear at the level of the CFU-GM. Thus, the therapeutic advantage we wish to exploit is based on our observation that clonogenic leukemia cells generally express the antigens we are targeting, whereas primitive normal progenitor cells do not.\textsuperscript{14} We are attempting to determine if leukemia cells from individual patients arise from a pluripotent progenitor cell population or from a later progenitor cell committed to the granulocyte/monocyte lineage. From the work of Fialkow and colleagues,\textsuperscript{27} examining clonality of various blood elements in patients with AML, one might predict that our treatment would be successful for some patients but not for patients whose cells had a more immature origin. Studies of the expression of cell surface antigens on AML cells and their progenitors have revealed that antigens are acquired in an orderly fashion.\textsuperscript{14,28} analogous to normal myeloid differentiation. Thus, it may be possible to correlate the antigenic phenotype of patients’ leukemia cells with the outcome of transplantation with MoAb-treated bone marrow in these patients. We examined the total leukemia cell population for antigen expression in the patients selected for this pilot study by flow cytometry and C\textsuperscript{-}dependent lysis. It may be more relevant to consider the phenotype of clonogenic leukemia cells. We were not able to measure the effect of MoAb therapy on clonogenic leukemia cells (L-CFC) from the patients reported in this study. We are encouraged, however, because a single exposure to MoAb PM-81 clone eliminated >50\% of the L-CFC from 13 to 20 patients with AML in another study, whereas MoAb AML-2-23 eliminated >50\% of L-CFC from 6 of the same 20 patients. This indicates that the antigens defined by these MoAbs are expressed at the level of the clonogenic leukemia cell in some patients with leukemia. The heterogeneity in antigen expression on L-CFC could allow some L-CFC to escape lysis, however. In a model system for clonogenic leukemia cells using the HL-60 leukemia cell line, we found that the degree of clonogenic cell kill can be increased by using two treatments rather than one\textsuperscript{18} and by using two MoAbs together (unpublished observations, June 1985). Thus, under such optimal conditions of C\textsuperscript{-}mediated lysis, the elimination of L-CFC is possibly greater than that predicted from the study by Sabbath and co-workers.\textsuperscript{14} Consequently, we are presently treating all bone marrows with both AML-2-23 and PM-81 whether the patients’ cells react with both MoAbs or only with PM-81. The ultimate success of this program depends on our ex vivo procedure, the efficacy of the preparative regimen, and the modern support services that have contributed greatly to improved survival of leukemia patients.

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REFERENCES

Autologous bone marrow transplantation in acute myelogenous leukemia: in vitro treatment with myeloid cell-specific monoclonal antibodies

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