Anti-CD33 Monoclonal Antibody and Etoposide/Cytosine Arabinoside Combinations for the Ex Vivo Purification of Bone Marrow in Acute Nonlymphocytic Leukemia

By Patrick J. Stiff, William C. Schulz, Michael Bishop, and Louis Marks

Pharmacologic and immunologic methods of ex-vivo bone marrow (BM) purging for acute nonlymphocytic leukemia (ANLL) (BM) were combined to augment the effect of either method alone. Etoposide (VP16; 20 to 30 μg/mL) with or without cytosine arabinoside (Ara C; 10 mg/mL) was used in tandem with the anti-CD33 monoclonal antibody (MoAb), MY9, chosen because CD33 is found on the stem cell pool in the majority of patients with ANLL. The agents were tested singly or sequentially, with a 1-hour incubation of the drugs preceding complement-mediated lysis using MY9. VP16 combined with Ara C killed up to 3.9 ± 0.3 and 5.1 ± 0.4 logs of the human ANLL cell lines HL60 and K562 at drug concentrations that killed only 1.2 ± 0.1 logs of normal committed granulocyte/macrophage stem cells (CFU-GM). Adding a single exposure of the MY9 and complement (C) to the drug-treated cells, greater than 5.4 logs of HL60 were killed. Similar to other pharmacologic agents, no differential kill for clonogenic leukemia cells (colony-forming unit-leukemia; CFU-L) from patients with ANLL was seen for drug only treated blasts versus normal CFU-granulocyte-macrophage (CFU-GM), with less than 1 log CFU-L kill at drug concentrations that spared 1 log of CFU-GM. Similarly, only 1.1 ± 0.3 logs of ANLL CFU-L were eliminated using MY9 and C'. However, with the sequential VP16/Ara C → MY9 + C' treatment, synergy was demonstrated and 2.6 ± 0.3 logs of CFU-L were eliminated. Because CD33 is also found on the normal CFU-GM pool, two-stage long-term BM cultures were performed to determine pluripotent stem cell elimination by the drug/MoAb purging combination. No difference of CFU-GM or BFU-E production at 4 to 6 weeks of culture for VP16/Ara C, MY9 + C', or VP16/AraC → MY9 + C' treated cells was seen compared with untreated controls indicating sparing of early progenitor cells. Sequential ex vivo treatment of human ANLL CFU-L with VP16/Ara C followed by complement-mediated lysis using MY9 synergistically kills CFU-L while sparing early normal hematopoietic progenitor cells, and thus may be a more effective way to purge BM than either alone.

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SUPRALETHAL chemoradiotherapy with an HLA-matched allogeneic bone marrow transplant (BMT) is curative therapy for selected patients with acute nonlymphocytic leukemia (ANLL). However, only 10% of patients with ANLL are eligible for this therapy because of age and donor restrictions. Supralethal therapy with an autologous BMT free of residual leukemic cells could also be curative and would have the advantages of not being complicated by graft versus host disease, would have less strict age requirements, and all patients who enter a remission would have a donor. Because residual ANLL cells are present in the majority of patients with a remission BM, pharmacologic and immunologic methods of purging these residual leukemic cells from autografted BM are being explored. While clinical trials of autologous BMT for ANLL are underway using either pharmacologic agents or monoclonal antibodies (MoAbs) to purge residual leukemic cells, neither method consistently kills greater than 1 log of ANLL clonogenic stem cells (colony-forming unit-leukemia; CFU-L) at doses that leave sufficient normal CFU-granulocyte-macrophage (CFU-GM) to reconstitute hematopoiesis after BMT. Thus, sufficient ANLL CFU-L are theoretically left to cause a relapse. While combinations of pharmacologic agents and MoAbs for purging remission BM of residual acute lymphocytic leukemia (ALL) cells have been shown to be synergistic, their use together has not been previously reported in ANLL. Based on current single-modality ANLL purging data, such a combination is possible. Etoposide (VP16), an effective agent for the treatment of ANLL has been investigated by our group for BM purging. VP16 is capable of killing both cycling and noncycling cells, and in a murine model, was shown not to cause a differential CFU-GM versus CFU-spleen (CFU-S) kill. In this model, up to 107 leukemia cells contaminating syngeneic BMT grafts were eliminated. Very high-dose cytosine arabinoside (Ara C; 10−2 mol/L) has also been investigated recently for BM purging because of the clinical effectiveness of high-dose Ara C treatment regimens, its lack of toxicity to the CFU-S pool in a murine model at these concentrations, and a suggested synergism with VP16. The mechanism of this synergism is unknown; however, Ara C may inhibit DNA strand breakage repair induced by VP16.

While no ANLL-specific myeloid MoAb exists, it is known that the cell of origin in the majority of cases of ANLL arises not at the CFU-S level but at the CFU-granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM) or early CFU-GM level of maturation, suggesting that myeloid MoAb expressed on both the CFU-GEMM and CFU-GM should be the primary MoAb used to purge BM of residual ANLL CFU-L. This finding is important because ANLL CFU-L are felt to have an antigenic expression more immature than that seen on the majority of leukemic blasts. The anti-CD33 myeloid MoAb MY9 was chosen for our studies because it is expressed on the CFU-GEMM, CFU-GM, as well as ANLL.

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CFU-L, but probably not on the CFU-S stem cell pool.

As the effectiveness of any ex vivo BM purging method depends not only on the amount of CFU-L kill, but also on the amount of normal pluripotent hematopoietic stem cell damage, it is necessary to define toxicity to normal stem cells. Because no direct assay for the human pluripotent stem cell is available, we investigated long-term marrow cultures (LTMC) to evaluate the effects of drug/MoAb purging on the pluripotent stem cell as has been previously done.

We report here our initial work of sequential ANLL purging using VP16/Ara C followed by complement-mediated lysis using MY9. Because of the discrepancies between leukemic cell line and CFU-L kill in our and others' previous studies for these agents, we included studies using leukemic blasts from patients with ANLL to determine efficacy. However, overall effectiveness was measured as a significant differential kill of the leukemic stem cell pool versus normal hematopoietic stem cells as measured by two-stage LTMC assays.

MATERIALS AND METHODS

Normal and leukemic cells. Normal human BM was aspirated from the iliac crests or sternum from normal allogeneic BMT donors after informed consent was obtained. Cells were aspirated into syringes containing preservative free heparin (Squibb, Cherry Hill, NJ). Mononuclear cells were recovered after separation using Ficoll-Paque (1.077 g/cm³) (Pharmacia, Piscataway, NJ).

HL60 promyelocytic and K562 erythroleukemia human cell lines (American Type Culture Collection, Rockville, MD) were maintained in suspension in log phase of growth. They were grown in RPMI-1640 (M.A. Bioproducts, Walkersville, MD) supplemented with 20% calf serum (Hyclone, Logan, UT) (20% RPMI) and 1% penicillin-streptomycin-neomycin (PSN) antibiotic solution (GIBCO, Grand Island, NY) at 37°C in a humidified atmosphere of 5% CO₂.

BM from patients with ANLL either at the time of diagnosis or relapse was aspirated from the posterior iliac crests after informed consent was obtained. Peripheral blood leukemic cells were used in patients with high circulating numbers of blasts. Relapsed patients had not received chemotherapy for at least the previous 8 weeks. After separating the mononuclear fraction, T lymphocytes were removed by rosetting with 2-amino-ethylisothiouronium bromide-treated sheep erythrocytes. Cells not used immediately were cryopreserved in 5% dimethyl sulfoxide (DMSO) and 6% hydroxyethyl starch and stored at -80°C as previously described.

In vitro colony assays. Normal BM CFU-GM assays were done as modified Pike and Robinson assays. Briefly, cells were cultured in quadruplicate in 35-mm plates (Falcon, Oxnard, CA) at 2 x 10⁵ cells/mL in 1% methylcellulose with McCoy’s 5A medium (M.A. Bioproducts) and 20% heat-inactivated fetal bovine serum (FBS) (Hazeltown, Lenexa, KS) over 1- to 7-day-old pre-established WBC feeder layers as above. They were cultured in quadruplicate at varying cell concentrations up to 2 x 10⁶ cells/mL at the predetermined concentration of the maximal cloning efficiency to generate less than 100 colonies per culture. K562 and HL60 clonogenic cells (tumor CFU; +CFU) giving rise to colonies of greater than 20 cells per aggregate were counted after 7 days in culture.

ANLL cells were cultured in quadruplicate at varying cell concentrations up to 2 x 10⁶ cells/mL in 0.8% methylcellulose with α-Minimal Essential Media (α-MEM; GIBCO) and 20% heat-inactivated FBS and 10% medium conditioned by leukocytes in the presence of 1% phytohemagglutinin (Wellcome Labs, Dartford, England), and incubated at 37°C in a humidified atmosphere at 5% CO₂ over pre-established irradiated WBC feeder layers as above. ANLL CFU-L giving rise to colonies of greater than 20 cells were counted after 7 to 14 days in culture.

Pharmacologic agents/MoAb. VP16 (20 mg/mL) (Bristol-Myers, Evansville, IN) was diluted in RPMI-1640 and bulk Ara C (Upjohn, Kalamazoo, MI) was reconstituted in acidic sterile distilled water immediately before each experiment. The anti-CD33 IgG, complement fixing myeloid MoAb MY9 (Coulter Immunology, Hialeah, FL) was used. Neonatal rabbit serum was used as a source of complement (C³) (Peltfreeze, Brown Deer, WI). Each lot was assayed for cytolytic activity and toxicity to normal BM cells. ANLL and HL60 cells were analyzed for MY9 positivity after labeling with the phycoerythrin conjugate MY9-RD1 (Coulter Immunology) and analyzed by flow cytometry, using an FITC 2 fluorescence-activated cell sorter (Becton Dickinson, Mountain View, CA). Patients with ANLL were considered to be positive for MY9 if greater than 20% of blasts were positive for MY9. The HL60 cell line was used as a control for the ANLL assays, with greater than 99% cells positive for MY9.

Purging studies. In vitro pharmacologic studies were performed using normal BM mononuclear cells, human ANLL cells, or HL60 and K562 cells. Cells were adjusted to 5 x 10⁶/mL and incubated with the drug(s) for 1 hour at 37°C with agitation in a water bath. Cells were then washed twice at 4°C and assayed for CFU-GM, BFU-E, ANLL CFU-L, or tCFU as described above.

For in vitro MoAb studies, BM mononuclear cells, MY9 positive ANLL cells, or HL60 cells were added to Hank's Balanced Salt Solution (M.A. Bioproducts) with 10% heat-activated FBS. MY9 was added to the cell pellet at 5 µg/10⁶ cells and incubated at 4°C for 30 minutes. Cells were washed once to remove excess antibody, and the cell count was adjusted to 5 x 10⁵ cells/mL. Rabbit C' was added at a final concentration of 1:4 for 1 hour at room temperature. Cells were agitated every 15 minutes, washed, and assayed as described above.

For the combined drug/MY9 purging studies, cells were treated sequentially with combinations of VP16 with or without Ara C for 1 hour, washed twice, and then incubated with MY9 + C' and assayed as described above.

LTMC. To determine pluripotent stem cell damage caused by the purging treatments, two-stage allogeneic LTMC were used. LTMC adherent layers were established using 20 x 10⁶ unfractionated normal BM cells in McCoy’s 5A media, supplemented with 12.5% heat-inactivated FBS, 12.5% heat-inactivated horse serum, 1% MEM vitamins, 1% penicillin, 1% glucose-serine-asparagine, 1% pyruvate, 0.8% essential amino acids, 0.4% nonessential amino acids, and 10⁻⁴ mol/L hydrocortisone sodium succinate as previously described. Cells were placed in 25-cm² tissue culture flasks (Corning, Corning, NY) at 37°C, allowed to equilibrate with 5% CO₂ for 2 hours, and the caps were then sealed tightly. Cultures...
were maintained by replacing half of the media and nonadherent cells weekly and subsequently maintained at 33°C after 1 week.

For the LTMC assays of purged BM, 20 to 40 × 10⁶ treated or untreated fresh mononuclear BM cells were placed over the pre-established 3 to 4-week-old allogeneic LTMC adherent layers that had been irradiated with 950 cGy using a Cs¹³¹ source. The flasks were incubated at 33°C, allowed to equilibrate with 5% CO₂ for 2 hours, and the caps were then sealed tightly. Cultures were maintained weekly with removal of half the supernatant media and nonadherent cells after gently shaking the flask and fresh LTMC media was replaced. Cells were counted, checked for viability by Trypan blue dye exclusion test, and assayed for CFU-GM or BFU-E. The assays were performed in 1% methylcellulose over WBC underlayers in modified McCoy's 5A media supplemented with 0.8% essential amino acids, 0.4% nonessential amino acids, 1% glutamine, 1% serine/pyruvate/asparagine, 1% PSN solution, and 10% heat-inactivated FBS. Colonies of greater than 40 cells were counted after 10 days in culture.

Significance was evaluated by the Student's r-test. Differences between groups were considered significant if P values were below .05.

RESULTS

Effect of VP16 and Ara C on CFU, CFU-L, and CFU-GM. After incubation with VP16, up to 3.1 ± 0.1 and 3.5 ± 0.3 logs of K562 and HL60 tCFU, respectively, were eliminated as shown in Table 1. Ara C alone at 10 mg/mL eliminated 0.8 ± 0.1 and 1.9 ± 0.3 log of K562 and HL60 CFU, respectively. While the addition of Ara C to VP16 significantly increased K562 tCFU log kill (P < .05), it did not increase the HL60 tCFU log kill. The Ara C/VP16 combination did not significantly increase normal BM CFU-GM kill over that seen with VP16 alone, with a maximal 1.2 ± 0.1 log kill for the 30 μg/mL VP16 with 10 mg/mL Ara C concentrations. Studies using VP16 at concentrations >40 μg/mL alone or in combination with Ara C resulted in less than 5% CFU-GM recovery compared with controls. Based on our previous data in a murine model that showed a nonselective CFU-GM versus CFU-S kill for VP16* and data showing a decreased survival for patients if less than 5% CFU-GM remain after BM purging with activated cyclophosphamide derivatives,²² we chose to investigate only drug/MoAb combinations using less than 40 μg/mL of VP16.

While there was a marked differential HL60 and K562 tCFU versus CFU-GM to VP16 alone or with Ara C, there was less than 1 log maximal ANLL CFU-L kill seen when fresh human ANLL cells were treated with these agents as shown in Table 2 with no differential CFU-L versus CFU-GM kill seen. Purging concentrations of VP16 ≥40 μg/mL alone or with Ara C also failed to show any differential ANLL CFU-L versus CFU-GM kill (data not shown).

Effect of MY9 on tCFU, CFU-L, and CFU-GM. Treatment of HL60 cells with MY9 + C’ resulted in a 2.7 ± 0.2 HL60 tCFU log kill. The ANLL samples used in this study were all MY9 positive, with a mean percent positive for separated blasts of 51% (range 28% to 72%). When assayed after treatment with MY9 + C’ alone 1.1 ± 0.3 log kill of ANLL CFU-L was seen, as shown in Table 2. As expected, MY9 + C’ eliminated 93.1% ± 2.1% of normal CFU-GM. Thus, no differential ANLL CFU-L versus CFU-GM kill was seen. No significant loss of HL60 tCFU, CFU-GM, or ANLL CFU-L was seen when MY9 alone or complement alone were used compared with untreated controls (data not shown).

Effect of sequential use of VP16 + Ara C followed by MY9 + C’ on tCFU and CFU-L. To determine if the combination of drugs and MoAbs were additive or synergistic, they were tested initially using HL60 targets. A significant increase in HL60 tCFU kill was seen for the sequential drug(s) → MY9 + C’ combination compared with either modality alone as shown in Table 3. The addition of MY9 + C’ to VP16 at 20 μg/mL resulted in 5.4 ± 0.4 log kill of HL60 tCFU. No growth was seen at higher doses of VP16 combined with MY9 + C’, or for VP16 plus Ara C with MY9 + C’ at either 20 or 30 μg/mL of VP16.

When ANLL cells were treated, the sequential addition of MY9 + C’ to VP16-treated cells caused an additive kill of ANLL CFU-L, over either modality alone as analyzed by the fractional product method. However, unlike the data showing no additional ANLL CFU-L kill when Ara C is added to VP16, when MY9 + C’ is sequentially added to the two-drug combination, there was a synergistic 0.65

### Table 1. Survival of K562, HL60 tCFU, and Committed Hematopoietic Stem Cells CFU-GM Purged With VP16 and Ara C

<table>
<thead>
<tr>
<th>Purging Doses</th>
<th>% Survival*</th>
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<tbody>
<tr>
<td>VP16 (μg/mL)</td>
<td>Ara C (mg/mL)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
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<tr>
<td>0</td>
<td>10</td>
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*Expressed as mean % (±SE) compared with untreated controls.
additional log kill of CFU-L, with a total of 2.6 ± 0.3 log kill seen for the VP16/Ara C → MY9 + C' combination, as shown in Table 4.

Effect of sequential use of VP16 + Ara C followed by MY9 + C' on CFU-GM and BFU-E in LTMC. To determine if the synergistic effect of this drug/MoAb combination caused additional normal hematopoietic stem cell inhibition over that caused by either modality alone, two-stage allogeneic LTMC assays were used (Table 5). For flasks containing VP16/Ara C only or MY9 + C' only treated cells, despite the survival of only 13.2% ± 4.5% and 6.9% ± 2.1% of CFU-GM, respectively, at inception of cultures, by week 4 of the LTMC assays, 105.43% ± 65.7%, and 103.87% ± 33.17% CFU-GM of untreated control cells, respectively, were produced (Fig 1A and B). Despite weekly demi-depopulation, CFU-GM could be detected at control levels at 6 weeks in LTMC when the cultures were discontinued. Irradiated control flasks did not produce significant numbers of CFU-GM or BFU-E.

For the combined sequential therapy, only 1.7% ± 1.1% and 1.9% ± 0.8% of control CFU-GM remained on the day the LTMC were initiated for the VP16 → MY9 + C' and VP16/Ara C → MY9 + C' flasks, respectively. However, by week 4 in LTMC, 119.7% ± 66.1% and 96.3% ± 58.6% CFU-GM were generated, respectively, compared with untreated control flasks (Fig 1C). In addition, the total number of CFU-GM generated per flask was similar to those containing VP16/Ara C or MY9 + C' only treated BM cells.

Compared with untreated controls, 23.0% ± 16.4% and 2.7% ± 1.4% of control BFU-E survived treatment with MY9 + C' and VP16/Ara C → MY9 + C' treatment, respectively (Table 5). By week 3 in LTMC cultures, BFU-E generated from the drug and drug/MoAb-treated cells were 187.4% ± 111.9% and 200% ± 100.9% of control flasks. In fact, while the flasks containing the treated cells continued to generate BFU-E at weeks 5 and 6, the control flasks characteristically did not. CFU-GEMM assays were also performed; however, too few colonies grew in the control flasks to draw any conclusions.

DISCUSSION

Patients with ANLL who do not have an allogeneic donor could undergo an autologous BMT with curative intent if the autograft contained insufficient viable clonogenic leukemic cells to cause a relapse. While several groups have investigated the use of unpurged remission BM for autotransplantation in ANLL, only 5% of patients transplanted at the time of relapse, and 25% of those transplanted within 4 months of achieving a first complete remission appear to be long-term survivors, ie, they have a prognosis similar to those treated with conventional chemotherapy. These poor results may be due in part to the fact that ANLL remission BM may still contain up to 10⁹ to 10¹⁰ BM leukemic cells. Because 1%–2% of the total BM is removed during a BM harvest, as many as 10⁶ to 10⁷ leukemic cells may contaminate the harvested BM, which appears to be sufficient to cause a relapse. For this reason, transplants using BM purged ex vivo are being investigated.

Autologous BMT purged with the activated cyclophosphamide derivatives 4-hydroperoxycyclophosphamide (4-HC) and mafosfamide (Asta Z) were the first such transplants performed. While initial results using 4-HC were encouraging, a recent update indicated that only 17.5% of patients transplanted in second or third complete remission have remissions that last longer than the first remission obtained using conventional chemotherapy. For purged autologous BMT with Asta Z, an improvement in disease-free survival (DSF) was reported for patients undergoing BMT in first, but not second remission. However, those patients transplanted longer than 4 months after achieving their first remission did not benefit from the transplant.

While these results suggest a benefit for subsets of patients with ANLL, as the prognosis of these patients is superior to those treated with conventional chemotherapy, longer follow-up is needed. While relapses may be occurring because of inadequate myeloablative preparative regimens, recent data testing the effectiveness of these agents in the elimina-

Table 3. Survival of HL60 cCFU Purged With VP16 Alone or With Ara C Followed Sequentially With MY9 + C’

<table>
<thead>
<tr>
<th>Purging Doses</th>
<th>% Survival*</th>
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<tbody>
<tr>
<td>VP16 (µg/mL)</td>
<td>Ara C (mg/mL)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
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<tr>
<td>20</td>
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*Expressed as mean % (±SE) compared with untreated controls.
Table 5. Mean % Colonies Generated in Two-Stage LTMC After Treatment With VP16 Alone or With ARA C Followed Sequentially With MY9 + C' Compared With Untreated Controls

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Week</th>
<th>CFU-GM (N = 6)</th>
<th>BFU-E (n=4)</th>
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<tbody>
<tr>
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<td></td>
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<tr>
<td>VP16/Ara C/MY9 + C'</td>
<td>0/0/0</td>
<td>(100.0 ± 15.2251.13)*</td>
<td>(3013.25 ± 899.61)*</td>
</tr>
<tr>
<td></td>
<td>20/0/0</td>
<td>16.18 ± 7.52</td>
<td>19.02 ± 10.31</td>
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<tr>
<td></td>
<td>20/10/0</td>
<td>13.18 ± 4.45</td>
<td>18.11 ± 5.84</td>
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<tr>
<td>0/0/MY9 + C'</td>
<td>0/0/0</td>
<td>6.85 ± 2.12</td>
<td>15.39 ± 5.09</td>
</tr>
<tr>
<td>20/0/MY9 + C'</td>
<td>1.72 ± 1.11</td>
<td>3.96 ± 1.86</td>
<td>17.90 ± 6.21</td>
</tr>
<tr>
<td>20/10/MY9 + C'</td>
<td>1.69 ± 0.78</td>
<td>41.46 ± 20.90</td>
<td>26.80 ± 8.93</td>
</tr>
</tbody>
</table>

*Values in parenthesis represent total number of CFU-GM or BFU-E per flask.
†Expressed as mean % (SE) compared with untreated control.
‡VP16 dose (20 μg/mL); Ara C dose (10 mg/mL).

Fig 1. Mean % CFU-GM generated in two-stage LTMC for BM treated with (A) VP16 alone ( — ) or in combination with Ara C ( — ), (B) MY9 + C' ( — — ), and (C) VP16 alone ( — — — — ) or combination with Ara C ( — — — — — ) followed sequentially with MY9 + C'.

In an attempt to improve upon these results, we have evaluated VP16 alone or in combination with other agents. Very high-dose Ara C, ie, concentrations 3 logs higher than that achieved in vivo with high-dose Ara C regimens, was tested because of the clinical efficacy of high-dose regimens, its sparing of the pluripotent pool in a murine model, and its possible synergism with VP16, by inhibiting repair of DNA damage caused by VP16. While synergism was seen using K562 as the cell target in our studies, no such effect was seen for either HL60 or ANLL clonogenic cells. In addition, VP16 was no better than the activated cyclophosphamide derivatives at eliminating ANNL CFU-L.

Consistent with animal models, the amount of clonogenic leukemic cell kill appears to correlate with the probability of relapse for patients undergoing chemoimmunopurged autografts for ALL. Patients autotransplanted with BM in which 2.0 ± 0.3 logs of CFU-L were eliminated remained in remission compared with a 0.9 ± 0.5 log kill for patients who relapsed after their BMT. As most agents and combinations fail to eliminate more than 1.0 to 1.5 log kill of CFU-L, suggesting inadequate purging as well.

While preclinical models demonstrate synergism when pharmacologic and immunologic methods of BM purging are combined for the typically homogenous forms of ALL until recently it has not been felt that MoAb purging of ANLL would be possible due to the lack of leukemia specific antigens and the overlap of antigen expression between ANLL cells and normal myeloid stem cells. However, recent data have shown that the cell of origin in ANLL arises at either the CFU-GEMM or early CFU-GM level of maturation in the majority of cases, with CFU-L having a maximal 1 to 1.5 log kill of CFU-L, suggesting inadequate purging as well.
were to be used to purge BM of residual ANLL CFU-L, MoAbs that recognize antigens expressed early in stem cell maturation would be the most beneficial. Antibodies that mark the primitive stem cell antigens such as CD34 or HLA-DR would not be necessary to purge ANLL CFU-L and would, in fact, be contraindicated, due to their demonstrated toxicity to the pluripotent stem cell pool. While antibodies such as PM-81, AML-2-23, and MY9 appear to be appropriate to consider for purging, our data and that previously reported demonstrate at best a 1 log CFU-L kill for each of these MoAbs. For our combination studies, the anti-CD33 myeloid MoAb MY9 was chosen because it is expressed on up to 90% of ANLL as well as ANLL CFU-L, and is also expressed on 95% CFU-GM and greater than 85% of CFU-GEMM. In addition, based on our LTMC data, it appears that the CD33 antigen is not expressed on the pluripotent stem cell pool. This confirms the data for the IgM anti-CD33 MoAb, L4F3.

While no additional ANLL CFU-L kill was seen with the addition of Ara C to VP16 in our studies, its addition to VP16 followed by purging with MY9 + C led to 1 additional log kill of CFU-L. This unexpected but consistent finding was also seen for the HL60 cell line studies. This result may be due in part to the fact that Ara C in high doses affects the phase equilibrium of the cell membrane by interaction with cell membrane components and inhibition of phospholipase A2. In addition, Ara C may inhibit fusion of fatty acid containing ternary mixtures. By affecting membrane structure and function, the cytolytic toxicity of complement may have been enhanced by the prior exposure to the Ara C.

By using a sequential drug/MoAb regimen, which has been reported to be effective for purging BM in ALL, we have demonstrated for the first time a more than additive kill of ANLL CFU-L compared with either method alone, with up to 2.7 ± 0.3 logs kill. In addition, this result was accomplished at drug concentrations that do not produce any inhibition of CFU-GM or BFU-E generation in LTMC. Based on engraftment data from our ongoing phase II clinical trial using VP16/Ara C for the ex vivo purging of BM and our LTMC data, we would predict engraftment if the combined modality therapy of VP16/Ara C plus MY9 + C were to be used for the ex vivo treatment of BM for patients with ANLL. This drug/MoAb combination may also be effective for the in vivo treatment of ANLL, if the recently initiated clinical trials of MY9-Ricin are positive.

Before initiating a clinical trial of this purging method several issues need to be considered. First, as not all ANLL is MY9 positive, combinations rather than single myeloid MoAb may be more appropriate to use for optimal purging. Second, because of the well-described inefficiencies of complement-mediated lysis, such as ineffective lysis of low antigen density cell populations, alternate methods of removing MoAb-coated cells from BM suspensions such as immunoadsorption or immunomagnetic separation deserve study. Finally, it will be necessary to determine if the amount of ANLL CFU-L kill seen here reflects the elimination of leukemic stem cells capable of self-renewal or just a committed stem cell population. The second plating efficiency (PE2) of leukemic blasts in vitro may be more predictive of the self-renewal capability of leukemic clonogenic cells than CFU-L; however, too few leukemic colonies, including an absence of all detectable colonies in one half of our samples, remained after the combined chemosorption and purging performed in this study to determine its merit. A leukemic suspension assay like that for normal stem cells would be of value. However, several groups have, in fact, shown that unpurged leukemic cells will often not proliferate in standard LTMC media, making analysis of purging data using this assay suspect. This difference between normal and leukemic cells has even led to a clinical trial with the autotransplantation of unpurged BM from patients with ANLL in remission or relapse after 10 days in LTMC media. The recently available recombinant colony-stimulatory factors may ultimately permit the development of a suspension assay capable of measuring the self-renewal capability of leukemic stem cells. Such an assay would have value not only in purging studies but also in the development of more effective conventional chemotherapy treatment regimens for patients with this disease.

**ACKNOWLEDGMENT**

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CHEMOMMUNO PURGING IN ANLL


Anti-CD33 monoclonal antibody and etoposide/cytosine arabinoside combinations for the ex vivo purification of bone marrow in acute nonlymphocytic leukemia

PJ Stiff, WC Schulz, M Bishop and L Marks