Utilization of a Colony Assay to Assess the Variables Influencing Elimination of Leukemic Cells From Human Bone Marrow With Monoclonal Antibodies and Complement

By Tucker W. LeBien, Daniel E. Stepan, Richard M. Bartholomew, Robin C. Stong, and Janet M. Anderson

We have previously used a chromium-release assay to demonstrate that the cocktail of monoclonal antibodies BA-1, BA-2, BA-3, and complement can effectively lyse human leukemic cells in the presence of excess bone marrow. Using a leukemic cell colony assay, we have reinvestigated the variables influencing lysis of human leukemic cells (KM-3, HPB-NULL, NALM-6) in bone marrow using BA-1, BA-2, BA-3, and complement. Specific variables addressed included the concentration of excess bone marrow cells, the number of treatments, the presence or absence of DNase during the treatment, the combination of antibodies, and the sensitivity of different leukemic cell lines to lysis. Using the colony assay, the BA-1,2,3 cocktail was shown to be more effective than any single antibody or combination of two antibodies. We also determined that the concentration of excess bone marrow cells and number of treatments had a direct bearing on leukemic cell lysis. Although two cycles of treatment were significantly superior to one cycle, three cycles were not significantly superior to two cycles. Inclusion of DNase (10 μg/mL) was a critical adjunct that eliminated clumping and facilitated plating cells in the colony assay. Finally, we could show that striking differences existed in the sensitivity of the leukemic cell lines to lysis with the BA-1,2,3 cocktail and complement. NALM-6 cells were the most sensitive (approximately four logs of kill), and KM-3 cells were the most resistant (less than two logs of kill). Our results strongly support the utility of sensitive leukemic cell colony assays in the analysis of marrow treatment variables in autologous bone marrow transplantation.

A VARIETY of strategies are currently being considered for the ex vivo elimination of neoplastic cells from autologous bone marrow grafts using monoclonal antibodies. The major objective inherent to all strategies is to effectively eliminate neoplastic cells, while sparing the hematopoietic stem cells crucial for engraftment. Clinical trials are being initiated at numerous centers, with most of the trials using monoclonal antibodies and exogenous complement (C'). Two reports on these preliminary trials have been recently published.

Our approach to the ex vivo elimination of leukemic cells in autologous bone marrow transplantation has drawn on our past experience in producing monoclonal antibodies that recognize cell surface molecules expressed on human acute lymphoblastic leukemia (ALL) cells. The monoclonal antibodies BA-1, BA-2 (anti-p24), and BA-3 (anti-glycoprotein [gp] 100/ common acute lymphoblastic leukemia antigen [CALLA]) are excellent reagents for ex vivo treatment of bone marrow based on (1) their binding to the vast majority of non-T ALL, and (2) their absence of reactivity with hematopoietic stem cells. We have presented preliminary data on the efficacy of BA-1,2,3 plus C' for eliminating residual leukemic cells in excess bone marrow using a 51Cr-release assay. We have also initiated a clinical trial in which bone marrow from pediatric ALL patients who failed conventional therapy has been treated with BA-1,2,3 plus C'.

In this report, we describe a leukemic cell line colony assay, developed and used to facilitate a more critical analysis of leukemic cell lysis with BA-1,2,3 plus C'. This assay is considerably more sensitive than the previously used 51Cr-release assay, and we report herein on the variables influencing effective lysis with our BA-1,2,3 antibody cocktail.

MATERIALS AND METHODS

Cells

The human ALL cell lines KM-3,13 HPB-NULL,14 and NALM-615 were maintained as suspension cultures in RPMI 1640 (GIBCO, Grand Island, NY) containing 5% fetal bovine serum (FBS, GIBCO) as previously described.16 These cell lines have cell surface characteristics of B cell precursors, but differ with respect to expression of cytoplasmic IgM (HPB-NULL and NALM-6, positive; KM-3, negative) and CALLA (vide infra). Bone marrow was procured from normal volunteers or transplant donors through the University of Minnesota Bone Marrow Transplant Program. Bone marrow was separated on Ficoll-Hypaque gradients, and the interface cells were suspended in RPMI 1640 containing 5% FBS at 1 to 2 x 10^5 cells per milliliter. Interface cells received 3,000 rad of gamma irradiation in a Mark I cesium irradiator.

From the Department of Laboratory Medicine and Pathology, University of Minnesota Medical School, Minneapolis, and Hybritech Incorporated, San Diego.

Supported by grant Nos. CA-21737 and CA-31685 from the National Cancer Institute, National Institutes of Health, and Biomedical Research Support grant No. RR-05385. T.W.L. is a Scholar of the Leukemia Society of America. D.E.S. was the recipient of a Zagaria Fellowship in Oncology.

Submitted April 16, 1984; accepted Oct 20, 1984.

Address reprint requests to Dr Tucker W. LeBien, Box 609 Mayo, Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55455.

© 1985 by Grune & Stratton, Inc.
Antibodies and Complement

Monoclonal antibodies BA-1, BA-2, and BA-3 were originally produced and characterized in this laboratory. Purification protocols were developed at Hybritech, San Diego. For use in this study, the antibodies were initially purified from ascitic fluid by precipitation with 18% sodium sulfate. BA-1 was then dialyzed against distilled water to obtain a euoglobulin precipitate. The BA-1 euoglobulin precipitate and the sodium sulfate precipitates of BA-2 and BA-3 were purified by diethylaminoethyl (DEAE) anion exchange chromatography using phosphate gradients for elution. Purity of the antibodies was checked by gel electrophoresis, and they were stored at 0.5 mg/mL with 2 mg/mL human serum albumin added for stabilization. BA-1, BA-2, and BA-3 were used at a final concentration for 10 μg/mL, unless otherwise indicated. This concentration saturated all available antigenic sites on leukemic cells in preliminary fluorescence-activated cell sorter (FACS) experiments. A hybridoma cell line secreting W6/32, an antibody recognizing a nonpolymorphic epitope on human class I HLA-A, B, C gene products, was obtained from the American Type Culture Collection (Rockville, Md). The cells were injected into pristane-primed mice to obtain ascitic fluid-derived W6/32, and the ascitic fluid was used at a final dilution of 1:100.

Baby rabbit complement (C) was obtained from Pel-Freez Biologicals, Rogers, Ar. C was passed through a sterile 0.22-μm filter and stored at −70 °C. Two separate lots of C were used during the course of the experiments described herein. Both lots were initially screened for cytotoxic capability in a 51Cr-release assay. Lot 0614 gave maximal killing at a final dilution of 1:3, whereas lot 0105 gave maximal killing at a final dilution of 1:5.

Treatment of Cell Mixtures

Cultures of KM-3, NALM-6, or HPB-NULL cells in log phase of growth were treated with various combinations of antibodies plus C' in the presence or absence of X-irradiated bone marrow cells, depending on the experiment. In preliminary experiments excluding X-irradiated bone marrow cells, KM-3 cells (final concentration of 5 x 10⁶ cells per milliliter) in RPMI 1640 containing 5% FBS were treated with antibodies plus C' or C' alone, in 12 x 75 polystyrene tubes (Falcon, Oxnard, Calif). The total reaction volume was 0.5 mL. After 70-minute incubation at 37°C in a 5% CO₂, humidified atmosphere, the treated cells were diluted and plated in the colony assay described below.

Experiments using X-irradiated bone marrow cell, leukemic cell mixtures were treated at a final ratio of 95% X-irradiated bone marrow cells/5% leukemic cells. Cell mixtures at the desired concentration were treated with antibodies plus C' or C' alone, in 12 x 75 polystyrene tubes. In experiments utilizing excess bone marrow and multiple cycles of treatment, deoxyribonuclease I (DNase, from bovine pancreas, Sigma Chemical Co, St Louis) was added to a final concentration of 10 μg/mL. The total reaction volume was either 0.3 or 0.5 mL. Cell suspensions were incubated at 37°C in a 5% CO₂, humidified atmosphere for the specified time, and the cells were gently resuspended every ten to 15 minutes. If the cell mixtures were to be treated with more than one antibody plus C', or C' alone, they were centrifuged for 1.5 minutes at high speed on a Serofuge (Clay Adams, Parsippany, NJ). The supernatant was then discarded, and the pelleted cells were resuspended to the exact same initial volume and concentration in additional medium, antibodies, and C'. After the last cycle of treatment, the cell mixtures were diluted and plated in the colony assay described below.

Colony Assay for Leukemic Cell Lines

Our colony assay for human leukemic cell lines is based, in part, on the assay described by Izaguirre and his colleagues. In order to simplify the assay, we initially screened a number of cell lines for their ability to form colonies in the absence of exogenous feeder cells and/or conditioned media. KM-3, NALM-6, and HPB-NULL were selected on this basis, and all three cell lines had a colony-forming efficiency of 8% to 40% in the presence or absence of excess X-irradiated bone marrow cells.

Leukemic cells treated with antibodies plus C' or C' alone, in the presence or absence of X-irradiated bone marrow cells, were then plated in the colony assay. An aliquot of treated leukemic cells, or leukemic cell/bone marrow cell mixtures, was diluted in minimum essential medium (MEM) (KC Biologicals, Lenexa, Kan) containing 10% FBS (HyClone, Logan, Utah). A 2.8% (wt/vol) methylcellulose (MC, MCB Reagents, Cincinnati) stock solution was then added to achieve a final concentration of 0.84% MC. The mixture was vigorously vortexed to thoroughly resuspend the cells and then maintained at 37°C in a 5% CO₂, humidified atmosphere for five to 15 minutes so that air bubbles that formed during the vortexing did not interfere with plating. The cells were distributed into 96-well flat-bottom microtiter plates (Costar, Cambridge, Mass) at 0.1 mL/well in replicates of six or more. The number of cells plated per well was based on the cell concentration before treatment, since the antibody plus C' treatment often completely lysed many of the cells. Final plating densities were selected to facilitate counting of colonies, and in general, wells were not counted if there were >100 colonies per well. Microtiter plates were incubated in a humidified Modulator Incubator Chamber (Billups-Rothenberg, Del Mar, Calif) flushed with a gas mixture consisting of 5% O₂, 5% CO₂, 90% N₂. The chamber was placed in a CO₂ incubator at 37°C. Colonies (>40 cells) were quantitated eight to 12 days later. The percentage suppression of colony formation was calculated by the formula:

\[
\frac{\text{No. colonies in C' control} - \text{No. colonies in experiment}}{\text{No. colonies in C' control}} \times 100\%.
\]

Statistical analysis of results obtained in the colony assay was performed using a one-tailed Student's t test. Our decision to analyze the data in one tail (as opposed to a two-tailed analysis), was based on previous data generated using 51Cr-release and trypan blue exclusion assays that showed that BA-1,2,3 plus C' was superior to any single antibody plus C'. We inferred that treatment with BA-1,2,3 plus C' would also be superior using the colony assay, and we proceeded to analyze the data in one tail to determine if the superiority was significant.

FACS Analysis

Flow cytometric analysis of immunofluorescent stained cells was conducted using a FACS-IV (Becton Dickinson, Mountain View, Calif) as previously described. Analysis was performed on 20 to 40,000 cells using the 488 nm line of a 5 W argon-ion laser. Data were transcribed from the FACS computer to an APPLE II computer for histogram production.

RESULTS

Figure 1 shows representative FACS histograms of KM-3, NALM-6, and HPB-NULL cell lines stained with BA-1, BA-2, and BA-3 by indirect immunofluorescence. Slight, apparently random differences (<5%) in the percentage of positive cells with a single antibody were noted in the cell lines during the course of the experiments described herein. The phenotypes (KM-3=BA-1', BA-2', BA-3'; NALM-6=BA-1', BA-2', BA-3'; HPB-NULL=BA-1', BA-2', BA-3') were, however, consonant with data previously reported from this laboratory.
Before using the human leukemic cell line colony assay as a modus operandi for analyzing elimination of residual leukemic cells by BA-1,2,3 plus C', it was essential to demonstrate that our culture conditions were optimal for colony formation. Figure 2 shows the linear relationship (through the origin of the plot) obtained when the mean number of HPB-NULL colonies per well was plotted as a function of the number of HPB-NULL cells initially plated per well. Such a linear relationship is consistent with optimal conditions for colony growth. It is worth emphasizing that linearity was achieved without exogenous conditioned media, without feeder cells, and in the presence of excess X-irradiated bone marrow cells. Linear growth in the presence of excess marrow was also achieved using KM-3 and NALM-6 cells (data not shown).

In a preliminary report we used a 51Cr-release assay to demonstrate that the BA-1,2,3 cocktail plus C' was more effective than any single antibody plus C' in lysing leukemic cells.11 As a starting point, therefore, we re-addressed this question with the colony assay using the treatment conditions (one cycle of treatment for 70 minutes) found to be optimal in the 51Cr-release assay. The results of several experiments using KM-3 cells are presented in Table 1. The conclusion reached from these experiments is that the BA-1,2,3 cocktail plus C' was significantly more effective than any single antibody, or combination of two antibodies plus C', with the marginal exception of BA-1,2 plus C' (P = .059). All antibodies were used at conditions of saturation shown to be 10μg/mL by FACS analysis. Increasing the antibody concentration did not enhance cell lysis (data not shown). In control experiments, incubation of cells with antibody alone had no effect on colony formation. Coupled with the FACS data shown in Fig 1, it is also apparent that a considerable number of KM-3 cells were not lysed in some experiments. Possible explanations for these results are addressed later in this report.

We next investigated the influence of excess marrow cell concentration and number of antibody plus C' treatments on KM-3 cell colony formation. In preliminary experiments, we noted that multiple antibody plus C' treatments resulted in significant clumping of the marrow/leukemic cell mixtures. We found that inclusion of DNase during the treatments was a criti-
Table 3. Effect of Treatment Number on Suppression of KM-3, NALM-6, and HPB-NULL Leukemic Cell Colony Formation by BA-1,2,3 Plus C’

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>KM-3</th>
<th>HPB-NULL</th>
<th>NALM-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 × 90 min</td>
<td>94.6</td>
<td>94.7</td>
<td>&gt;99.98</td>
</tr>
<tr>
<td></td>
<td>94.1</td>
<td>93.8 ± 1.0†</td>
<td>91.3</td>
</tr>
<tr>
<td></td>
<td>92.6</td>
<td>97.0</td>
<td>99.38</td>
</tr>
<tr>
<td>2 × 45 min</td>
<td>96.4</td>
<td>98.3</td>
<td>&gt;99.98</td>
</tr>
<tr>
<td></td>
<td>95.0</td>
<td>95.6 ± 0.7</td>
<td>99.4</td>
</tr>
<tr>
<td></td>
<td>95.4</td>
<td>97.6</td>
<td>&gt;99.98</td>
</tr>
<tr>
<td>3 × 30 min</td>
<td>96.2</td>
<td>98.1</td>
<td>&gt;99.98</td>
</tr>
<tr>
<td></td>
<td>96.0</td>
<td>96.0 ± 0.3</td>
<td>99.5</td>
</tr>
<tr>
<td></td>
<td>95.7</td>
<td>98.1</td>
<td>&gt;99.98</td>
</tr>
</tbody>
</table>

* Treatments were conducted with a constant ratio of 5% KM-3, HPB-NULL, or NALM-6/95% bone marrow cells for the indicated times.
† Mean ± standard deviation of percentage suppression for three experiments.
‡ Means and standard deviations could not be calculated because of assay limitations.

To achieve much greater than 95% suppression of KM-3 cell colony formation with up to three cycles of BA-1,2,3 plus C’. These results could be explained by the presence of a KM-3 subpopulation inherently resistant to C'-mediated lysis, or a KM-3 subpopulation weakly expressing one or more of the antigens recognized by BA-1, BA-2, and BA-3. Table 4 shows the results of an experiment to test the former possibility. Whereas one 70-minute treatment with BA-1,2,3 plus C’ resulted in 88% suppression, the same treatment with W6/32 plus C’ resulted in >99.98% suppression. We conclude that KM-3 cells did not contain a population of C’-resistant cells. We then considered the possibility that the resistant population had a lower antigen density. KM-3 cells were treated with three cycles of BA-1,2,3 plus C’, and control cells were treated with C’ alone. After removal of dead cells by Ficoll-Hypaque centrifugation, the viable interface cells were stained with BA-1,2,3 plus fluorescein isothiocyanate-goat anti-mouse Ig (FITC-GAM) or FITC-GAM alone, and analyzed on a FACS IV (Fig 3). It is apparent that KM-3 cells surviving three cycles of BA-1,2,3 plus C’ express smaller amounts of BA-1,2,3 antigens than cells treated with C’ alone (compare Fig 3A with 3C). It is also apparent that most of the cells surviving three cycles have antibody bound to their surface (compare Fig 3B with 3D). We conclude that the inability to kill more than 95% of KM-3 cells probably reflects escape from lysis due to low density expression of BA-1,2,3 antigens.

**DISCUSSION**

The utilization of monoclonal antibodies for the ex vivo elimination of residual malignant cells in autologous transplantation is being pursued with increasing vigor by a large number of laboratories. Our choice of...
antibodies for marrow treatment is unique, and we have discussed the reasoning for our selection in detail elsewhere.\textsuperscript{11,21} The data reported herein support the utility of a colony assay for analyzing variables influencing the lysis of leukemic cells by BA-1,2,3 plus C'. The assay is sensitive to a minimum of four logs of leukemic cell kill, and it is not dependent on exogenous conditioned media or feeder cells. Colony-forming efficiencies of 8\% to 40\% were achievable with KM-3, NALM-6, and HPB-NULL cell lines. This is equivalent or superior to results achieved with other leukemic cell lines in similar assays.\textsuperscript{21,22}

In a previous paper,\textsuperscript{11} we reported that treatment of leukemic cell/marrow mixtures for 70 minutes with BA-1,2,3 plus C' killed \textasciitilde95\% leukemic cells as measured by \textsuperscript{51}Cr-release. We and others\textsuperscript{21} have discussed the problems inherent to \textsuperscript{51}Cr-release, in particular the lack of sensitivity above 95\% lysis. As a result, we developed a colony assay to monitor for the presence of residual leukemic cells, and we used this assay to re-address some of the key variables influencing lysis with antibody and C'. As a starting point, we demonstrated that the BA-1,2,3 cocktail was superior to any single antibody or combination of two antibodies (Table 1). In agreement with Bast et al.,\textsuperscript{20} we have shown that the concentration of excess marrow cells and number of treatments does influence the efficacy of complement-mediated cytolysis. It is clear from Table 2 that lowering the marrow cell concentration to 1 \times 10^7 cells per milliliter and treating with two cycles of BA-1,2,3 plus C' gave superior killing. Unlike Bast et al.,\textsuperscript{20} we could not show that three cycles of treatment were significantly better than two cycles (Table 3). It is possible that the simultaneous analysis of three antibodies obscured our ability to detect a significant difference between two and three cycles of treatment.

The inclusion of DNase was a critical adjunct that minimized cell clumping and, therefore, facilitated plating cells in the colony assay. DNase probably digested nuclear DNA liberated from cells lysed during the treatment, thereby minimizing clumping.

Another conclusion drawn from our studies is the differing sensitivity of leukemic cell lines to lysis with BA-1,2,3 plus C'. We could not achieve two-log suppression of either KM-3 or HPB-NULL colony formation with up to three cycles of treatment. In the case of KM-3, this resistance to lysis was probably attributable to a minor population of cells that weakly expressed the antigens recognized by BA-1,2,3 (Fig 3), as opposed to the existence of a subpopulation of cells resistant to C'-mediated lysis (Table 4). In contrast to results obtained with KM-3 and HPB-NULL, the NALM-6 cell line was greater than two logs more sensitive to suppression of colony formation by BA-1,2,3 plus C'. The explanation for this difference in sensitivity is not clear, but may relate to the relative differences in antigen expression. The histograms in Fig 1 show that NALM-6 cells stain more brightly with BA-1, BA-2, and BA-3 compared with KM-3 and HPB-NULL cells.

Treatment of human bone marrow with the BA-1,2,3 cocktail plus C' is nontoxic to hematopoietic precursor cells. This has been documented in vitro using standard bone marrow colony-forming assays (CFU-E, BFU-E, CFU-GM, CFU-GEMM).\textsuperscript{11} Furthermore, bone marrow from 23 patients with ALL has been treated with BA-1,2,3 plus C',\textsuperscript{12,24} In no case to date has BA-1,2,3 plus C' treated and cryopreserved marrow failed to engraft.

It is worth noting that although difficult to quantitate, we may be achieving an increase in the therapeutic index using two-cycle treatment of bone marrow with BA-1,2,3 plus C'. Table 2 shows that two 35-minute treatments at 1 \times 10^7 cells per milliliter is vastly superior to one 70-minute treatment at 5 \times 10^7 cells per milliliter for killing of KM-3 leukemic cells. As part of our ongoing clinical trial, CFU-GM have been cultured from bone marrow treated under both the aforementioned conditions with no quantitative differences in colony growth.\textsuperscript{12,24}

<table>
<thead>
<tr>
<th>Table 4. Comparison of BA-1,2,3 Plus C' v W6/32 Plus C' on Suppression of KM-3 Leukemic Cell Colony Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>BA-1,2,3 + C'</td>
</tr>
<tr>
<td>W6/32 + C'</td>
</tr>
</tbody>
</table>

This experiment was conducted in the absence of excess bone marrow. KM-3 cells were treated with BA-1,2,3 plus C', W6/32 plus C', or C' alone for 70 minutes.

*Mean ± standard deviation of percentage suppression calculated from six-well replicates.
In conclusion, we have presented data documenting the utility of the BA-1,2,3 monoclonal antibody cocktail plus C' to eliminate residual leukemic cells in human bone marrow. Several different technologies are now available for eliminating contaminating neoplastic cells in autologous marrow grafts using monoclonal antibodies. Indeed, reports have recently appeared describing the utility of antibody plus C',20 antibody-ricin,21,22,25 and magnetic microspheres.26 No published studies have yet appeared comparing the efficacy of the various technologies. Whether BA-1,2,3 linked to ricin or 4-hydroperoxycyclobphosphamide is more effective than BA-1,2,3 plus C' is currently being investigated. Critical, comparative studies of the different technologies are now necessary to define a standard treatment protocol potentially applicable to larger numbers of patients.

ACKNOWLEDGMENT

The authors thank Mike Hupke for FACS analyses, Anne Goldman for assistance with the statistics, Norma Ramsay and Karin Smith for CFU-GM data, and Janelle Evavold for typing the manuscript.

REFERENCES

16. LeBien TW, Hurwitz RL, Kersey JH: Characterization of a xenotensismer produced against three molar KCL solubilized antigens obtained from a non-T, non-B (pre-B) acute lymphoblastic leukemia cell line. J Immunol 122:82, 1979

From www.bloodjournal.org by guest on August 30, 2017. For personal use only.
Utilization of a colony assay to assess the variables influencing elimination of leukemic cells from human bone marrow with monoclonal antibodies and complement

TW LeBien, DE Stepan, RM Bartholomew, RC Stong and JM Anderson