Elimination of Malignant Clonogenic Cells from Human Bone Marrow Using Multiple Monoclonal Antibodies and Complement

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ABSTRACT

A clonogenic assay has been developed that utilizes Burkitt's lymphoma tumor cell lines to detect elimination of up to 5 logs of tumor cell contamination within human bone marrow. Different Burkitt's lymphoma lines bear one or more of a group of markers, including common acute lymphoblastic leukemia antigen gp26 (glycoprotein with a molecular weight of 26,000), B1, surface membrane immunoglobulin, HLA, β2-microglobulin, and 1a. Burkitt's tumor cells of the Namalwa line have been mixed with a 20-fold excess of irradiated human bone marrow cells. After treatment with one or more monoclonal antibodies and rabbit complement (RC), mixtures have been grown on a monolayer of irradiated human bone marrow cells and tumor cells enumerated by limiting dilution. Multiple treatments with antibody and RC were more effective than a single treatment in destroying clonogenic tumor cells which bore relevant determinants. Human serum components inhibited the lytic activity of RC in the presence of murine monoclonal antibodies. The total concentration of bone marrow cells proved critical in determining the complete elimination of tumor. Incubation of the Namalwa tumor cell line with RC and the J2 anti-gp26 eliminated more than 3 logs of malignant cells from a 20-fold excess of human bone marrow. Combinations of two monoclonal antibodies were more effective than any single antibody in eliminating Namalwa cells. A combination of three monoclonal reagents was no more effective than a combination of J2 and B1 or J2 and J5 in eliminating Namalwa cells. Treatment of human bone marrow with three antibodies and RC did not, however, produce a selective loss of nonmalignant GM-CFU-C, CFU-E, or BFU-E.

INTRODUCTION

Autologous bone marrow transplantation might aid in management of leukemia and lymphoma if malignant cells could be removed quantitatively from normal hematopoietic precursors. Availability of essentially unlimited quantities of monoclonal antibody bodies with precisely defined reactivity and high titer has stimulated interest in immunoseparation of malignant and normal cells (2). In the case of ALL, the J5 monoclonal antibody reacts with CALLA, which is expressed by leukemic cells from approximately 70% of patients with non-T-cell disease (12). Although CALLA is expressed by 1% of normal marrow precursors, it is not found on GM-CFU-C, BFU-E, or bipotent granulocyte erythroblast precursors that can be measured in vitro (7). Treatment of marrow with anti-CALLA and RC prior to autologous bone marrow transplantation in patients who have received ablative therapy with total body irradiation and combination chemotherapy has not prevented engraftment, suggesting that CALLA is not expressed by the pluripotent stem cell (14). Ultimately, the utility of this approach will depend upon the elimination of leukemic cells from bone marrow both in vitro and in vivo. In earlier studies, we had defined requirements for eliminating a nonlymphocytic leukemia from rat bone marrow, using a polyclonal heteroantisemur and RC (8). A single treatment with heteroantisemur and RC could eliminate 10⁴ leukemic cells in the presence of 1.6 x 10⁵ nucleated bone marrow cells. Repeated treatment with heteroantisemur and RC could eliminate 10⁶ leukemic cells, provided that mature leukocytes and erythrocytes were removed on density gradients of Ficoll-diatriozate. Treatment of the rat marrow with heteroantisemur and RC did not prevent hematopoietic reconstitution of lethally irradiated recipients (8). A similar approach was extended to the elimination of ¹⁵¹Cr-labeled CALLA-positive NALM-1 cells from human bone marrow in vitro, using the monoclonal J5 antibody and RC (3). Optimal lysis of leukemic cells was obtained by treating ¹⁵¹Cr-labeled NALM-1 for a total of 90 min with the J5 monoclonal antibody and RC. Three treatments for 30 min were more effective than 2 treatments for 45 min or one treatment for 90 min. Separation of marrow on Ficoll-diatriozate was not essential, but did reduce the number of nucleated cells requiring treatment. Concentrations of bone marrow in excess of 2 x 10⁷/ml inhibited elimination of leukemic cells. Repeated treatment with J5 and RC, which eliminated greater than 99% of leukemic cells, produced a 50% loss of normal nucleated cells, but no selective loss of GM-CFU-C, BFU-E, or bipotent granulocyte-erythroblast colony-forming precursors (7).

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Despite these encouraging results, conventional assays of \(^{51}\)Cr release can measure only 2 logs of tumor cell elimination in vitro. Consequently, we have developed a more sensitive clonogenic assay which permits the detection of up to 5 logs of tumor cell elimination in vitro. Using this assay, we have reexamined requirements for eliminating malignant cells from bone marrow with a single monoclonal antibody and RC. In addition, it has been possible to ask whether multiple monoclonal antibodies against distinct determinants would prove more effective than a single reagent in eliminating malignant cells from human bone marrow.

**MATERIALS AND METHODS**

**Lymphoma Cell Lines.** The Namalwa cell line was used for these studies (4). This cell line expressed CALLA, gp26, and B1. Portions of malignant cells were cryopreserved in medium that contained 20% heat-inactivated FBS and 10% dimethyl sulfoxide. Cells were stored in the vapor phase of liquid nitrogen. Each 2 weeks cells were thawed rapidly and diluted slowly in MEM supplemented with 5% FBS without dimethyl sulfoxide. Cultures were prepared in RPMI Medium 1640 containing 10% heat-inactivated FBS, 1 mm sodium pyruvate, 2 mm L-glutamine, 10 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 100 units penicillin/ml, and 100 µg streptomycin/ml. Cultures were incubated at 37° in an atmosphere of 5% CO\(_2\)/95% humidified air, and were split 1:18 to 24 hr prior to each assay to assure that cells were proliferating optimally.

**Bone Marrow.** After obtaining informed consent under protocols approved by the Dana-Farber Human Protection Committee, bone marrow from healthy volunteers was collected in syringes that contained preservative-free heparin (Pan Heparin; Abbott Laboratories, N. Chicago, IL). Marrow was diluted 15-fold with Hank’s balanced salt solution without calcium or magnesium (M.A. Bioproducts, Walkersville, MD). Diluted marrow was layered over Ficoll-diatozite (Bionetics Laboratory Products, Kensington, MD), and centrifuged at 1300 x g for 15 min at 20°. Cells at the interface were washed twice in Hank’s balanced salt solution and once in MEM (M.A. Bioproducts) that contained Earle’s balanced salt solution, 2 mm L-glutamine, 10 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, and 5% FBS or 5% human AB serum that had been heat-inactivated at 56° for 30 min. When required, cells were sedimented at 200 x g for 10 min at 4°. After gradient separation and washing, the yield per aspiration was approximately 40 ± 10 (S.E.) x 10\(^6\) nucleated cells, of which greater than 98% were viable, judged by exclusion of 0.1% trypan blue. In some experiments whole heparinized human bone marrow was washed and used without gradient separation. After washing, the bone marrow cells were irradiated to 5000 R at 1390 R/min. Irradiated bone marrow cells were used both to prepare feeder layers and to prepare mixtures with nonirradiated tumor cells prior to treatment with monoclonal antibodies and RC. For studies of GM-CFU-C, CFU-E, and BFU-E, marrow was separated on Ficoll-diatozite and washed without irradiation.

**Monoclonal Antibodies.** All the monoclonal reagents utilized in these studies have been previously described in detail (9, 11, 13). J5 is an IgG2a murine monoclonal antibody that reacts with a glycoprotein with a molecular weight of 100,000 that is expressed in cells from 70% of patients with non-T-cell ALL and approximately 8% of patients with T-cell ALL (12, 13). J5 also reacts with cells from a fraction of patients with chronic myelogenous leukemia in blast crisis, and with virtually all tumors from patients with Burkitt’s lymphoma and nodular poorly differentiated lymphocytic non-Hodgkin’s lymphoma. J2 is an IgM immunoglobulin which reacts with gp26; gp26 can be detected in tumor cells from some patients with ALL, chronic lymphocytic leukemia, acute myelogenous leukemia, and Burkitt’s lymphoma (9). Anti-B1 is an IgG2a murine monoclonal antibody which reacts with a glycoprotein with a molecular weight of 35,000 found on the surface of normal B-cells and B-cell tumors (11). Approximately 50% of ALLs that express CALLA will coexpress B1. As a control, J0 has been obtained from mice inoculated with a hybridoma that produced IgG2a. It did not react with CALLA, gp26, or B1.

**Complement.** Different lots of RC with low titers of natural antibody against human bone marrow and Burkitt’s lymphoma tumor cell lines were obtained from Pelfreeze, Inc. (Glen Rock, NJ). RC was absorbed once at 4° with an equal volume of washed human AB erythrocytes and stored at −80° until use.

**Serial Dilution Assay.** Based upon experience in chromium release assays (3), mixtures of tumor cells and irradiated human bone marrow were incubated with monoclonal antibodies (1:100) for 15 min at 4° in a total volume of 1 ml MEM supplemented with 5% FBS. Incubation at low temperature minimized antigenic modulation of determinants such as CALLA. After incubation with antibody, RC (1:10) final dilution was added and the cells were further incubated for 30 min at 37° in total volume of 1.11 ml. The mixtures were then washed twice with MEM and 5% FBS. In most experiments, the cycle of antibody and RC treatment was repeated twice more, for a total of 3 cycles. Following the last incubation with RC, tumor cells were washed twice in clonogenic medium which contained RPMI medium 1640 with 10% heat-inactivated FBS, 1 mm sodium pyruvate, 2 mm L-glutamine, 100 units penicillin/ml, and 100 µg streptomycin/ml. A series of 12 serial 5-fold dilutions were prepared from suspensions that initially contained 10⁶ tumor cells/ml. From 6 to 10 aliquots (100 µl) of each dilution were plated in 96-well flat-bottomed microtiter plates that had already been seeded with 10³ irradiated bone marrow cells/100 µl of liquid clonogenic medium. Plates were incubated for 14 days at 37° in 5% CO₂/95% humidified air. Growth of colonies was scored visually and by labeling with [³H]thymidine. In preliminary studies, excellent agreement was obtained between scoring of cells visually and by radio labeling techniques.

**Statistical Analysis of the Clonogenic Assay.** Limiting-dilution analysis was performed using a Spearman estimate as described by Johnson and Brown (10). The mean of the dose-response function for each treatment was estimated by:

\[ m = x_o + \frac{d \cdot k \cdot n \cdot r_i}{2} \]  

where \( x_o = \) initial dose = 10⁻¹⁰; \( d = \) dilution factor = ln 5; \( n = \) number of wells at each dilution = 6 (in all but 2 experiments with 10); \( k = \) number of 5-fold dilutions = 9; and \( r_i = \) the number of wells with observed growth at the ith dilution. The estimated number of clonogenic units per ml was then calculated as \( m = \exp(-0.57722 - m) \). The initial dose (10⁻¹⁰) and the number of dilutions (k = 9) were chosen to have a high probability (>0.99) that \( n = \alpha \) and \( r_i = 0 \). Tests of significance to compare 2 treatments were based on the asymptotic normality of the estimates of the means (\( m_i \)), each with variance closely approximated by \( d \ln 2/n \). A Z statistic was used to estimate the level of significance.

A formal comparison was performed in which the means and variances were obtained from the Spearman estimator; 5-fold dilutions with 6 wells/dilution permitted the detection (at \( p = 0.05 \) 2-sided) of a 10-fold difference in clonogenic units between 2 treatment groups with probability 0.90. Corrections for multiple comparisons were made when appropriate.

**Clonogenic Assays of Normal Marrow Precursors.** Nonirradiated human bone marrow from normal donors was treated with monoclonal antibodies and RC, as described for the clonogenic assay of Burkitt’s cells. GM-CFU-C, CFU-E, and BFU-E were assayed as previously described (7).

**RESULTS**

**Optimal Antibody Concentration.** To determine the optimal concentration of J5, J2, or B1 antibody that would eliminate...
malignant cells from human bone marrow, 10^6 Namalwa cells were mixed with a 20-fold excess of irradiated human bone marrow and treated 3 times with absorbed RC and different dilutions of each monoclonal antibody. When the J5 anti-CALLA antibody was tested (Table 1), significant lysis was obtained at dilutions of each monoclonal antibody. When the J5 anti-CALLA marrow and treated 3 times with absorbed RC and different were mixed with a 20-fold excess of irradiated human bone marrow. When the J5 anti-CALLA or the J0 control antibody and RC. Incubation with the monoclonal antibody and RC was carried out in medium containing 10% human AB serum or 10% fetal calf serum (Table 2). In the presence of human AB serum, an estimated 2.6 x 10^6 clonogenic units remained after 1 or 3 treatments with J5 control antibody and RC. A single 90-min treatment with J5 and RC failed to reduce clonogenic units, and 3 treatments for 30 min reduced clonogenic units by less than 1 log. When the experiment was done in the presence of fetal calf serum, 2.0 x 10^6 clonogenic units could be grown after a single treatment, and 6.7 x 10^4 after 3 treatments. Here, a single 90-min treatment with J5 monoclonal antibody and RC reduced clonogenic units by more than a log, and 3 treatments were significantly more effective, reducing clonogenic units by more than 3 logs relative to the J0 control in FBS, and by almost 4 logs relative to the J0 monoclonal antibody (1:100) and RC (1:10) in the presence of human AB serum or FBS. These data suggested that in the clonogenic assay, as in our earlier experiments, multiple treatments with a single monoclonal antibody and RC were more effective than a single treatment of comparable duration in eliminating malignant cells from human bone marrow. Moreover, human AB serum appeared to inhibit the action of RC on human cells in the presence of murine monoclonal antibody.

**Optimal Bone Marrow Concentration.** In studies with ^51^Cr-labeled CALLA-positive cells, the concentration of bone marrow proved critical for the apparently complete elimination of malignant cells. In the clonogenic assay, 10^6 Namalwa cells were incubated different concentrations of human bone marrow, ranging from 2 x 10^5 to 2 x 10^8 viable nucleated cells/ml. Concentrations of marrow in excess of 2 x 10^6/ml appeared to inhibit elimination of Namalwa cells (Table 3). In a subsequent experiment, the marrow concentration was varied from 1 x 10^6 to 2 x 10^7 cells/ml. Optimal elimination of clonogenic Namalwa cells was observed at a marrow concentration of 5 x 10^5/ml (Chart 1).

**Use of 1, 2 or 3 Monoclonal Antibodies.** In previous studies with the J5 monoclonal antibody and RC, 2 logs of ^51^Cr-labeled NALM-1 cells could be eliminated from a 20-fold excess of human bone marrow (3). Given the more sensitive clonogenic assay, it was possible to examine whether treatment with multiple monoclonal antibodies might provide more complete elimination of malignant cells than treatment with any single reagent. For these experiments, 10^6 Namalwa cells were mixed with a 20-fold excess of irradiated human bone marrow. The mixtures were treated 3 times for 30 min with J5, J2, and B1, alone or in combination. After treatment with the J0 control antibody and RC, 4.4 x 10^5 clonogenic units could be recovered (Table 4).

**Optimal Number of Treatments.** In studies with the Wistar-Furth nonlymphocytic leukemia in rats (8) and with ^51^Cr-labeled NALM-1 cells (3), multiple treatments were more effective than a single treatment with antibody and RC in removing malignant cells from bone marrow. To test this question in the clonogenic assay, 10^6 Namalwa cells were treated once for 90 min, or 3 times for 30 min with either the J5 anti-CALLA or the J0 control antibody and RC. Considering the greater effect of multiple antibodies, it seemed possible that we might be able to avoid multiple treatments with antibody and RC by using 2 or 3 reagents in combination. Similarly, the use of an optimal concentration of marrow might also reduce the requirement for multiple treatments with antibody and RC. Consequently, 10^6 Namalwa cells were mixed either with 5 x 10^5 or with 2 x 10^6 irradiated bone marrow cells. Mixtures were treated once or 3 times with RC and a control antibody (J0), or with a combination of J5, J2, and B1. When all 3 antibodies were used in combination in the presence of an optimal marrow concentration, 3 treatments for 30 min were still more effective than a single treatment for 90 min in eliminating clonogenic cells (Table 5). Approximately 3-fold greater elimination of lymphoma cells was observed with the optimal concentration of bone marrow, but the difference was not statistically significant.

**Washing of Cell Mixtures.** In previous studies, mixtures of malignant cells and bone marrow were washed between each treatment with antibody and RC. As this requires a substantial amount of time and results in the loss of a fraction of the normal...
bone marrow cells, assays were compared in which mixtures were either washed or not washed between multiple treatments with antibody and RC. No difference was observed with or without washing between treatments using J5 monoclonal antibody and RC (Table 6).

Effect of Treatment with Multiple Monoclonal Antibodies and RC on Normal Marrow Precursors. To permit effective autologous bone marrow transplantation, techniques that eliminate malignant cells must not destroy normal marrow precursors. Consequently, we have examined whether treatment with a combination of J5, J2, B1, and RC produces a selective elimination of GM-CFU-C, CFU-E, or BFU-E. As indicated in Table 7, 3 treatments with multiple monoclonal antibodies and RC did not produce a selective loss of these colony-forming precursors.

DISCUSSION

A clonogenic assay has been developed which permits detection of nearly 5 logs of tumor cell destruction in the presence of a 20-fold excess of irradiated human bone marrow. Using this assay, conditions have been defined for eliminating Burkitt’s lymphoma cells, using one or more murine monoclonal antibodies and absorbed RC. The concentration of antibody required for removal of clonogenic cells was similar to that observed for the elimination of 32Cr-labeled NALM-1 cells (3). Multiple treatments with antibody and RC proved more effective than a single treatment, even when multiple monoclonal reagents were utilized and when the concentration of bone marrow was optimized. As in earlier studies (3), normal bone marrow cells inhibited lysis of admixed malignant cells. The basis for this inhibition is unknown, although C1 inhibitors have been described among lymphoid cells (5). The effect of human AB serum is of particular interest. Preliminary studies suggest that human C1 inhibitor may be important, since inhibition of tumor cell lysis is not observed with serum from patients with angioedema who have a low level of C1 inhibitor. Complement is required for inhibition of clonogenic growth. Attempts to demonstrate antibody-dependent cell-mediated cytotoxicity against Namalwa cells have failed, using human peripheral blood mononuclear cells and the same monoclonal antibodies. Moreover, comparable elimination of clonogenic cells was obtained with antibody and RC in the presence and absence of irradiated bone marrow.

Combinations of 2 monoclonal antibodies proved more effective than a single monoclonal reagent for eliminating tumor cells. In murine studies, clones of tumor that failed to express a relevant antigen have evaded serotherapy in vivo (6). Preliminary data suggest that clones which survive treatment with the J5 antibody and RC are relatively deficient in expression of CALLA.7 Alternatively, binding of additional immunoglobulin to the tumor cell surface may provide additive or synergistic cytolytic activity, even when antigen expression is preserved. In the future, polyclonal reagents may be blended from individually active monoclonal components. It is apparent, however, that arbitrarily adding additional reagents may not improve elimination of malignant cells from human marrow. Three antibodies were not more effective than the optimal combination of 2 reagents. In this regard, the clonogenic assay might aid in predicting useful combinations of monoclonal antibodies.

Removal of 99.99% of malignant cells from human bone marrow at a concentration of 2 × 107/ml may prove sufficient for quantitative removal of leukemic cells in autologous bone marrow transplantation. This is, however, a model system, and tumor cells may not be eliminated as readily when taken directly from a patient. In many cases, the residual cells will be resistant to cytotoxic drugs, and it is not known whether this would affect
MARROW CLEANSING WITH MONOCLONAL ANTIBODY AND RC

Table 5

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Duration</th>
<th>Clonogenic units</th>
<th>5 x 10^6 marrow cells/ml</th>
<th>2 x 10^6 marrow cells/ml</th>
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<tr>
<td>J0 + RC</td>
<td>1 treatment for 90 min</td>
<td>1.5 ± 0.83 x 10^6</td>
<td>4.4 ± 2.40 x 10^6</td>
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<tr>
<td></td>
<td>3 treatments for 30 min</td>
<td>1.5 ± 0.97 x 10^6</td>
<td>2.6 ± 1.62 x 10^6</td>
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<tr>
<td>J5, J2, and B1 + RC</td>
<td>1 treatment for 90 min</td>
<td>3.9 ± 2.43 x 10^6 (65%64)</td>
<td>2.2 ± 0.82 x 10^6 (63%28)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 treatments for 30 min</td>
<td>2.0 ± 1.86 x 10^6 (10%99)</td>
<td>6.3 ± 2.89 x 10^6 (99%95)</td>
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</tr>
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* Mean ± S.E.

Table 6

<table>
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<tr>
<th>Washed between cycle</th>
<th>Antibody</th>
<th>Clonogenic units</th>
<th>% inhibition</th>
</tr>
</thead>
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<tr>
<td>+</td>
<td>J0</td>
<td>25.5 ± 1.39 x 10^6</td>
<td>99.6</td>
</tr>
<tr>
<td></td>
<td>J5</td>
<td>9.17 ± 5.23 x 10^6</td>
<td>99.9</td>
</tr>
<tr>
<td>-</td>
<td>J0</td>
<td>9.79 ± 6.01 x 10^6</td>
<td>99.8</td>
</tr>
<tr>
<td></td>
<td>J5</td>
<td>2.05 ± 1.68 x 10^6</td>
<td>99.8</td>
</tr>
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* Mean ± S.E.

Table 7

<table>
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<tr>
<th>Treatment</th>
<th>Colonies/10^6 bone marrow cells</th>
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<tbody>
<tr>
<td></td>
<td>CPU-E</td>
</tr>
<tr>
<td>Control</td>
<td>220.0 ± 42.5</td>
</tr>
<tr>
<td>J0 + RC</td>
<td>152.7 ± 8.0</td>
</tr>
<tr>
<td>J5, J2, and B1 + RC</td>
<td>246.0 ± 17.0</td>
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</table>

* Mean ± S.D.

their sensitivity to monoclonal antibody and RC. Cells in bone marrow may also be dormant, in contrast to the rapidly proliferating cells that are used in the present assay. On the other hand, these data may in fact reflect the situation in marrow taken directly from patients. Evaluation of this question will require development of more effective techniques for growing leukemia and lymphoma cells in vitro immediately after bone marrow aspiration.

Autologous bone marrow transplantation might prove useful in the management of leukemia and lymphoma, provided that disease can be treated effectively in vivo as well as in vitro. Recent studies with syngeneic (1) or autologous (14) transplants suggest that high doses of cyclophosphamide and total body irradiation may salvage a small fraction of patients with ALL or lymphoma in second or subsequent remission. The ultimate success of this approach will depend not only on complete, or near complete, elimination of malignant cells from human bone marrow in vitro, but also on developing more effective techniques for eliminating malignant cells from the remainder of the patient during ablative therapy. Alternatively, autologous bone marrow transplantation, if proven to be safe in Phase I trials, might be applied to patients during first remission, if patients with an appropriately poor prognosis could be identified. Autologous bone marrow transplantation offers a promising alternative to allogeneic bone marrow transplantation, in that autologous transplants might succeed in adults over age 30 and in patients who do not have histocompatible donors.

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