Elimination of Leukemic Cells from Human Bone Marrow Using Monoclonal Antibody and Complement

Robert C. Bast, Jr., Jerome Ritz, Jeffrey M. Lipton, Maryellen Feeney, Stephen E. Sallan, David G. Nathan, and Stuart F. Schlossman

ABSTRACT

Human leukemic cells which bear the common acute lymphoblastic leukemia antigen can be lysed with a murine monoclonal antibody (J-5) in the presence of rabbit complement. Conditions have been defined for eliminating 51Cr-labeled common acute lymphoblastic leukemia antigen-positive NALM-1 cells or cryopreserved leukemic lymphoblasts from a 100-fold excess of human bone marrow. Optimal lysis is obtained with treatment for a total of 90 min. Three treatments for 30 min are more effective than two treatments for 45 min or one treatment for 90 min. Separation of marrow on a Ficoll:diatrizoate gradient does not permit more effective elimination of leukemic cells. Tumor cell lysis is inhibited by high concentrations of common acute lymphoblastic leukemia antigen-positive cells (2 × 10^7/ml) and by high concentrations of bone marrow (10^8/ml). Under optimal conditions, >99% of 51Cr-labeled leukemic lymphoblasts can be eliminated from a 100-fold excess of human marrow. Selective removal of leukemic cells from human bone marrow in vitro should facilitate trials of autologous marrow transplantation.

INTRODUCTION

Autologous bone marrow transplantation might provide effective treatment for acute leukemia if malignant cells could be eliminated from marrow in vitro without destroying pluripotent stem cells. Syngeneic transplantation of marrow from healthy identical twin donors has permitted long-term disease-free survival in 20 to 30% of recipients with acute leukemia (6). Promising results have also been obtained with syngeneic bone marrow transplantation in patients with lymphoma (1).

Studies in animal systems suggest that leukemic cells might be eliminated selectively by incubating marrow with complement and antibodies directed against tumor-associated antigens that are not expressed on pluripotent stem cells (4, 5, 18). Attempts have been made to eliminate ALL cells from human bone marrow using conventional heteroantisera (11, 19), but the development of monoclonal reagents should permit more effective and consistent treatment of marrow that is contaminated with leukemic cells. One particularly promising reagent is the J-5 murine monoclonal antibody that reacts with CALLA, a M, 100,000 glycoprotein (14) that is expressed on neoplastic cells from a majority of patients with ALL, chronic myelogenous leukemia in blast crisis, nodular non-Hodgkin’s lymphoma, T-cell lymphoblastic lymphoma, and Burkitt’s lymphoma (13, 14). The antigen can also be detected in 1 to 3% of cells from regenerating bone marrow (7) but is not associated with granulocyte-macrophage colony-forming cells, burst-forming units (erythrocytes), or mixed granulocytopoietic colonies (3). Use of J-5 for serotherapy in vivo has been limited by modulation of CALLA from the surface of leukemic cells (15) and by a failure of human complement to mediate tumor cell lysis (16). J-5 is, however, an IgG2 immunoglobulin that will lyse CALLA-positive cells in vitro in the presence of rabbit complement. Consequently, we have defined conditions under which J-5 could be used to eliminate CALLA-positive cells from human bone marrow.

Our previous studies with a rat model suggested that multiple treatments with a conventional heteroantisera and complement would provide more effective elimination of leukemic cells than would a single treatment (5). Removal of mature granulocytes and erythrocytes on density gradients might also facilitate the selective destruction of malignant cells (5). Particular attention has been paid to the possibility that a similar strategy would permit optimal utilization of monoclonal antibody and complement to eliminate leukemic cells from human marrow.

MATERIALS AND METHODS

Bone Marrow. After obtaining informed consent, bone marrow from healthy volunteers was collected in syringes that contained preservative-free heparin (Panheparin; Abbott Laboratories, North Chicago, Ill.). Care was taken to aspirate no more than 2 ml of marrow from any given site. Marrow was diluted 15-fold with HBSS (M. A. Bioproducts, Walkersville, Md.) without calcium or magnesium. Diluted marrow was layered over Ficoll:diatrizoate gradient (15, 19). Cells at the interface were washed twice in HBSS and once in MEM (M. A. Bioproducts) which contained Earle’s balanced salt solution, 2 mM L-glutamine, 12 mM HEPES buffer, and 5% human AB serum that had been heat inactivated at 56° for 30 min. For use, cells were sedimented at 200 × g for 10 min at 4°. After washing, cells were resuspended in MEM (M. A. Bioproducts) which contained Earle’s balanced salt solution, 2 mM L-glutamine, 12 mM HEPES buffer, and 5% human AB serum that had been heat inactivated at 56° for 30 min. For use, cells were sedimented at 200 × g for 10 min at 4°. After washing, cells were resuspended in MEM (M. A. Bioproducts) which contained Earle’s balanced salt solution, 2 mM L-glutamine, 12 mM HEPES buffer, and 5% human AB serum that had been heat inactivated at 56° for 30 min. For use, cells were sedimented at 200 × g for 10 min at 4°. After washing, cells were resuspended in MEM (M. A. Bioproducts) which contained Earle’s balanced salt solution, 2 mM L-glutamine, 12 mM HEPES buffer, and 5% human AB serum that had been heat inactivated at 56° for 30 min. For use, cells were sedimented at 200 × g for 10 min at 4°. After washing, cells were resuspended in MEM (M. A. Bioproducts) which contained Earle’s balanced salt solution, 2 mM L-glutamine, 12 mM HEPES buffer, and 5% human AB serum that had been heat inactivated at 56° for 30 min. For use, cells were sedimented at 200 × g for 10 min at 4°.
Leukemic lymphoblasts were thawed rapidly immediately prior to use, diluted gradually with 10 volumes of MEM supplemented with 5% human AB serum, and washed twice in the same medium prior to labeling. The NALM-1 cell line was derived from the peripheral blood lymphocytes of a patient with chronic myelogenous leukemia in blast crisis (10). NALM-1 cells are CALLA positive and have been maintained in Roswell Park Memorial Institute Tissue Culture Medium 1640 (M. A. Bioproducts) supplemented with 2 mM L-glutamine, 1 mM pyruvate, penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum. Cells were washed twice in MEM with 5% human AB serum prior to labeling. After washing, NALM-1 cells and thawed lymphoblasts were consistently >95% viable by exclusion of 0.1% trypan blue.

Lymphoblasts, NALM 1 cells, and bone marrow cells were labeled by incubating 2 × 10⁷ cells with 200 μCi sodium [51Cr]chromate (New England Nuclear, Boston, Mass.) in a total volume of 0.4 ml for 30 to 45 min at 37° with occasional agitation. After incubation, labeled cells were washed 3 times in MEM supplemented with 12 mM HEPES buffer and 5% human AB serum. Although the number of cpn incorporated by targets in different experiments varied with different batches of 51Cr, comparable amounts of radioactivity were incorporated by lymphoblasts and normal nucleated bone marrow cells (8,075 ± 2,185 cpm/10⁶ lymphoblasts and 10,385 ± 1,453 cpm/10⁵ bone marrow cells over 9 representative experiments). NALM-1 cells took up approximately twice as many counts (22,252 ± 5,065 cpm/10⁶ NALM-1 cells). Each of the different cell types, however, exhibited a similar ratio of spontaneous release to maximal release of counts after freezing and thawing labeled cells (28.0 ± 14.4, 29.5 ± 10.2, and 31.4 ± 17.0 for lymphoblasts, marrow, and NALM-1, respectively). In each case, the number of cpn remaining in the cell pellet after 10⁵ labeled cells had been frozen, thawed, and washed exceeded the machine background by at least 10-fold.

Complement. Healthy adult New Zealand White rabbits were bled aseptically. Blood was kept to clot for 1 hr at 37°, and clots were allowed to retract for an additional 2 hr at 4°. Serum was separated by centrifugation and stored for 1 hr at 0° with an equal volume of human AB erythrocytes that had been washed twice with HBSS prior to use. Preliminary experiments indicated that a single absorption with human AB erythrocytes was as effective as a single absorption with agarose or sequential absorption with erythrocytes and agarose for removing antibodies that could mediate lysis of human bone marrow. Only rabbits with low titers of antibody against human marrow were chosen as complement donors. Following absorption, complement was prepared, aliquoted, and stored at −80° until use.

Antibody. Preparation of the J-5 monoclonal immunoglobulin has been described (8). The hybridoma has been grown as ascites in BALB/c mice. A control ascites, J-0, has been obtained from mice inoculated with a hybridoma that produced an IgG2 that did not react with CALLA. Ascites containing either J-5 or J-0 were centrifuged twice at 1300 × g for 15 min to remove intact cells. Supernatant ascites was passed through filters of progressively finer porosity, finally including a Nalgene filter of 0.2 μm (Nalgene/Sybron, Rochester, N. Y.). After filtration, J-5 and J-0 were stored at −80° until use. Antibody titer was assessed conveniently and, in fact, represented the material that would actually be returned to a transplant recipient. Lysis therefore was measured routinely by 51Cr associated with the cell pellet following treatment with J-5 and complement, J-0 and complement, or with medium that lacked these reagents. The percentage of lysis of cells was calculated by comparison with 51Cr counts that remained associated with the cell pellet after washing. No significant difference was encountered when cells were frozen and thawed 1 or 6 times. As a single cycle produced >99% loss of viability judged by total cell count and exclusion of 0.1% trypan blue, controls were frozen and thawed once for the studies described below. All assays were performed in duplicate or triplicate. Significance of differences between groups was evaluated statistically using the t test.

RESULTS

Optimal Antibody Concentration. To determine the optimal concentration of specific monoclonal antibody that would prime CALLA-positive cells for complement-mediated lysis, 10⁵ NALM-1 cells were incubated with different dilutions of J-5 prior to a single incubation for 90 min with rabbit complement. J-5 can mediate lysis of NALM-1 at a dilution of 1:10,000 (Chart 1). The addition of a 100-fold excess of normal bone marrow had only a modest effect on the lysis of NALM-1 cells over a broad range of antibody concentration. Greater than 90% release of label could be achieved with antibody dilutions ranging from 1:10 to 1:1000. A dilution of 1:100 was chosen for subsequent experiments.

Optimal Duration of Complement Treatment. Using optimal concentrations of J-5 (1:100) and complement (1:10), the
duration of incubation with rabbit complement was varied from 0 to 180 min (Chart 2). Optimal lysis of $10^5$ NALM-1 was observed at 90 min following the addition of complement. As additional antibody-mediated lysis of NALM-1 was not observed after 90 min, this interval was chosen for further study. Data in Chart 2 also underline the specificity of J-5 which failed to lyse labeled human marrow. Control ascites (J-O) failed to produce $^{51}$Cr release from either NALM-1 or human bone marrow cells in the presence of complement.

**Optimal Number of Treatments.** Previous studies with a rat model suggested that multiple treatments with antibody and complement might prove more effective than a single treatment for eliminating leukemic cells from bone marrow. Having documented optimal lysis of NALM-1 following 90-min incubation with complement, a single treatment for 90 min was compared to 2 treatments for 45 min or 3 treatments for 30 min. Three treatments with J-5 and complement proved superior to one or two treatments for eliminating $10^5$ NALM-1 from a 100-fold excess of human marrow (Table 1). When NALM-1 was incubated with J-5 and complement in the absence of marrow, 3 treatments were significantly more effective than a single treatment, but the difference between 2 and 3 treatments was less marked. Either in the presence or in the absence of marrow, >99% lysis of NALM-1 was observed only after 3 treatments. This may relate in part to the nonspecific loss of cells by repeated washing. However, as the quantitative elimination of leukemic cells may be critical to the success of autologous marrow transplantation, conditions that produce >99% lysis are of particular interest.

**Separated versus Nonseparated Marrow.** In the rat model, separation of marrow of FD gradients prior to the addition of leukemic cells permitted more effective elimination of malignant cells using a heteroantiserum and complement. To determine whether gradient separation of human marrow would facilitate elimination of CALLA-positive leukemic cells using J-5 and complement, marrow was obtained from normal donors and portions of the marrow separated on FD gradients. In 5 replicates determinations using marrow from different donors, NALM-1 was eliminated as effectively from nonseparated as from separated bone marrow (Table 2).

**Concentration of CALLA-positive Cells and Bone Marrow Cells.** The ability of J-5 and complement to eliminate NALM-1 depended upon the concentration of CALLA-positive cells and the concentration of normal marrow. When $2 \times 10^3$ to $2 \times 10^6$ NALM-1 cells were incubated with J-5 and complement 3 times for 30 min, >95% of labeled cells were lysed (Chart 3). With $2 \times 10^3$ NALM-1 cells/ml, J-5 and complement lysed only 80% of the labeled targets. Similar results were obtained in the presence or absence of $2 \times 10^7$ gradient-separated bone marrow.

---

**Table 1**

<table>
<thead>
<tr>
<th>Treatment (min)</th>
<th>NALM-1</th>
<th>NALM-1 + Bone Marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x 90</td>
<td>96.0</td>
<td>91.7</td>
</tr>
<tr>
<td></td>
<td>94.5</td>
<td>93.0 ± 9.3</td>
</tr>
<tr>
<td></td>
<td>94.0</td>
<td>93.5</td>
</tr>
<tr>
<td>2 x 45</td>
<td>95.4</td>
<td>87.0</td>
</tr>
<tr>
<td></td>
<td>96.6</td>
<td>94.0 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>97.1</td>
<td>91.4</td>
</tr>
<tr>
<td>3 x 30</td>
<td>98.9</td>
<td>99.4</td>
</tr>
<tr>
<td></td>
<td>99.5</td>
<td>98.3 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>101.2</td>
<td>99.6</td>
</tr>
</tbody>
</table>

*Data from 3 experiments performed on 3 different days.

---

**Table 2**

<table>
<thead>
<tr>
<th>Marrow donor</th>
<th>Nonseparated</th>
<th>Gradient separated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>89.5</td>
<td>92.7</td>
</tr>
<tr>
<td>2</td>
<td>99.0</td>
<td>92.4</td>
</tr>
<tr>
<td>3</td>
<td>96.0</td>
<td>96.5 ± 4.0*</td>
</tr>
<tr>
<td>4</td>
<td>99.2</td>
<td>99.2</td>
</tr>
<tr>
<td>5</td>
<td>97.2</td>
<td>95.4 ± 2.8</td>
</tr>
</tbody>
</table>

*Mean ± S.D.
marrow cells/ml. Provided that the total concentration of NALM-1 did not exceed $10^7$/ml, addition of up to 50% labeled or nonlabeled NALM-1 to normal marrow failed to inhibit tumor cell lysis (Table 3).

Elimination of NALM-1 was also studied in the presence of a 100-fold excess of gradient-separated human bone marrow, maintaining a constant ratio of tumor cells to bone marrow. In the absence of marrow, similar lysis was observed over the entire range of NALM-1 concentrations from $5 \times 10^3$ to $1 \times 10^6$/ml (Chart 4). However, when $10^6$ marrow cells were added to $10^6$ NALM-1 cells/ml, lysis was inhibited.

The effect of marrow concentration was further evaluated by adding different numbers of bone marrow cells to a constant number ($10^5$) of NALM-1 cells/ml. Inhibition of NALM-1 lysis was observed in the presence of $10^8$ marrow cells/ml (Chart 5), in agreement with earlier studies at a lower marrow:target cell ratio (Chart 4). As less than 2% of normal marrow cells were CALLA positive by indirect immunofluorescence, $10^8$ marrow cells would contribute less than $2 \times 10^6$ CALLA-positive cold targets, which should not inhibit tumor cell lysis (Chart 3). Consequently, it appears that, at a sufficiently high concentration, CALLA-negative cells in normal marrow can inhibit NALM-1 lysis.

Elimination of Cryopreserved Leukemic Cells from Human Marrow. Similar observations have been made when J-5 and complement have been used to treat mixtures containing human bone marrow and cryopreserved CALLA-positive leukemic lymphoblasts. Three treatments with J-5 and complement provided more effective elimination of labeled cells than did one or two treatments (Table 4). Under optimal conditions, three 30-min treatments with J-5 (1:100) and absorbed rabbit complement (1:10) produced >99% lysis of $51^Cr$-labeled leukemic lymphoblasts in the presence of a 100-fold excess of gradient-separated human marrow with an average loss of approximately one-half of nucleated cells (Table 5).

**DISCUSSION**

Taken together with our previous observations (3, 17), conditions have been defined for eliminating >99% of leukemic cells from a 100-fold excess of human bone marrow without affecting the clonogenicity of the marrow in vitro or its ability to engraft in vivo. Treatment of marrow on 3 occasions with J-5...
antibody and complement reduced the total number of nucleated cells by 50% but failed to produce any selective loss of granulocyte-macrophage colony-forming cells, granulocyte-macrophage colony-forming erythrocytes, burst-forming units (erythrocytes), or bipotent granuloerythropoietic colony-forming precursors (3). In addition, 3 patients with CALLA-positive ALL in second or third remission have undergone autologous marrow transplantation following ablation with VM-26, 1-β-D-arabinofuranosylcytosine, cyclophosphamide, and 850 rads of total-body irradiation (17). Autologous marrow from each of the 3 patients had been treated with J-5 antibody and complement using the conditions found optimal in the studies described above. As anticipated, a 50% loss of nucleated elements was observed during treatment with J-5 and complement, but it was still possible to reinfuse 2 x 10^7 viable cells/kg body weight. Engraftment of granulocytes has been observed in each of the 3 patients. One of the 3 patients relapsed 2 months after transplantation, before reconstitution of all elements could occur. Full reconstitution of granulocytes (>3000/cu), erythrocytes (hematocrit, >36), and platelets (>200,000) has been observed in the remaining 2 recipients who are disease free 20 and 22 months following transplantation. Consequently, it appears that CALLA is not expressed on the pluripotent stem cell required for reconstitution of hematopoietic elements.

Although other investigators have used conventional heteroantisera in an attempt to purge human marrow of leukemic cells in the past (11, 19), monoclonal reagents now provide a virtually unlimited source of antibodies that have a reproducible pattern of reactivity and that do not require absorption. Given the relatively large amounts of antibody required to treat dilute marrow, a relatively large number of potential transplant candidates, and an absolute requirement for antibody specificity, monoclonal antibodies may well facilitate trials of autologous marrow transplantation. Our study is the first systematic attempt to define optimal conditions for utilizing a monoclonal antibody to eliminate leukemic cells from human bone marrow. As other reagents become available, similar studies will have to be undertaken before they can be utilized most effectively in the clinic. Optimal conditions may vary between antibodies, but experience with J-5 should prove useful in identifying at least some of the relevant variables which affect the susceptibility of malignant cells to treatment with antibody and complement in the presence of normal hematopoietic elements.

In previous clinical studies, marrow has been treated once with conventional heteroantisera and complement (11, 19). In murine and rat models for autologous marrow transplantation (4, 5, 18), a single treatment with heteroantisera and complement has removed approximately 2 logs of tumor in the presence of marrow, judged by the ability of treated cells to produce leukemia following inoculation into syngeneic recipients. Using a rat model (5), it has been possible to modify techniques for the treatment of marrow to permit elimination of at least 3 logs of leukemic cells. Repeated treatment was more effective than a single incubation with conventional heteroantisera and complement. Three incubations for 30 min proved optimal, provided that mature myeloid and erythroid elements were eliminated from marrow on FD gradients prior to the addition of the leukemic cells.

Data presented above suggest that repeated treatment of human bone marrow containing leukemic cells with antibody and complement should prove more effective than a single treatment. In contrast to work in the rat system, however, separation of marrow on FD gradients did not facilitate removal of leukemic cells with J-5 and complement. This may relate to the extraordinarily high titer of the monoclonal reagent compared to that of a conventional antiserum. Alternatively, mature myeloid and erythroid elements in rat marrow may exert more potent anticomplementary activity than do similar elements in human marrow. Whether or not gradient separation facilitates elimination of leukemic cells, reduction of erythrocyte contamination does minimize release of free hemoglobin from cryopreserved marrow after thawing. In addition, gradient separation reduces the number of nucleated cells by approximately 50%.

In both the animal and human systems, lysis of 51Cr-labeled leukemic cells was inhibited at high marrow concentrations. A C1 inhibitor is produced by splenic mononuclear cells, and it is possible that similar cells are present in the marrow (2). Whatever the underlying mechanism, it appears that optimal elimination of leukemic cells will be observed when marrow concentrations do not exceed 2 to 4 x 10^7 nucleated cells/ml (Charts 3 and 4). Consequently, rather large volumes of relatively dilute marrow must be processed to provide sufficient cells to reconstitute the lethally irradiated host. Availability of high-titered monoclonal reagents in very large quantities should permit treatment of marrow at an optimal cell concentration. Availability of large quantities of absorbed rabbit complement poses a logistical problem that deserves further attention if this approach to autologous bone marrow transplantation is to be utilized in a larger number of medical centers.

Use of antibody:toxin conjugates might provide an alternative to antibody and complement for the elimination of leukemic cells in vitro. One group has reported recently the rapid elimination of BCI-1 leukemia cells from murine bone marrow using an antiimmunoglobulin:ricin A chain conjugate (8). Our own data with a J-5:ricin A chain conjugate indicate that prolonged incubation with conjugates for as long as several days may be required to kill CALLA-positive cells (12). These observations have discouraged us from the use of a J-5:ricin chain conjugate to remove malignant cells from human bone marrow, but other toxins might prove more valuable.

Using 51Cr release as an index of leukemic cell death, destruction of only 2 logs of tumor cells can be measured with certainty. Three treatments of human marrow with J-5 and complement may eliminate a larger or a smaller fraction of the clonogenic subpopulation. In the W/Fu rat model, 91% lysis of leukemic cells judged by 51Cr release in vitro resulted in the elimination of at least 3 logs of tumor cells following transplantation in vivo (5). More effective suppression of tumor growth in vivo may relate to destruction of antibody-coated tumor through antibody-dependent cell-mediated cytotoxicity, complement-mediated cytotoxicity, or phagocytosis by cells of the transplant recipient (4, 5). Alternatively, clonogenic cells may be unusually susceptible to damage by antibody and complement, or lethally damaged cells may not release 51Cr promptly. The latter possibilities could be evaluated in the case of human bone marrow by developing clonogenic assays for CALLA-positive leukemia or lymphoma cells. Availability of these assays, if sufficiently sensitive, may permit detection of greater than 2 logs of tumor cell killing, permitting studies which combine J-5 and complement with other agents and techniques that eliminate leukemic cells from human marrow. Clonogenic
assays would also permit detection of CALLA-negative tumor cells that might escape treatment with J-5 and complement.

Treatment of marrow with monoclonal antibodies in vitro avoids some of the difficulties encountered when serotherapy is administered directly to patients. Heterologous complement can be used. Extracellular antigen can be washed from the marrow prior to treatment with antibody and complement. A large excess of antibody can be added to the system, and the marrow concentration can be varied. Antibodies, such as J-5, which bind to nonmyeloid tissues in vivo, can be used in vitro provided that the antibody does not react with pluripotential marrow stem cells. This may be particularly important in the case of J-5, as CALLA has been detected in normal adult kidneys (9). Modulation of certain tumor-associated antigens, such as CALLA, can occur in vivo as well as in vitro. Conditions can, however, be chosen in vitro which will minimize the effect of modulation. Attempts can also be made to modify the susceptibility of tumor targets to antibody-dependent lysis, using drug concentrations or conditions of incubation that would not be tolerated by tissues outside the bone marrow. Ultimately, the success of autologous marrow transplantation will depend upon the ability to eliminate residual tumor within the patient as well as within the bone marrow. Neoplasms that respond to total-body irradiation or to high doses of drugs that exert only myelotoxicity are still the most probable targets for autologous marrow transplantation. These diseases include the acute leukemias, chronic myelogenous leukemia in blast crisis, and the lymphomas. As CALLA is associated with several of these cancers, J-5 is likely to facilitate the autologous marrow transplantation of diseases other than ALL.

ACKNOWLEDGMENTS

We thank Mitchell Weiss for valuable technical assistance.

REFERENCES


Elimination of Leukemic Cells from Human Bone Marrow Using Monoclonal Antibody and Complement

Robert C. Bast, Jr., Jerome Ritz, Jeffrey M. Lipton, et al.