Blood, Vol. 55, No. 4 (April), 1980

Investigation of the Blastogenic and Cytotoxic Capabilities of Human Bone Marrow: Differences Between Aspirate and Rib Marrow

By Charles D. Alley and Richard P. MacDermott

The use of marrow aspirates in bone marrow transplantation, clinical evaluation, and experimental studies has prompted the present study of the functional characteristics of mononuclear cells from aspirated (iliac crest) and curettaged (rib) marrow. In this manner, the relative contribution of peripheral blood contamination of aspirated bone marrow cells in investigating and understanding marrow function can be assessed. Human bone marrow specimens were collected using 3–5 ml aspirates from the iliac crest in 17 individuals and using curettage of ribs obtained from 13 individuals undergoing thoracotomy. The marrow samples were separated over discontinuous Ficoll-Hypaque gradients. Mononuclear cells from aspirated marrow had a high background stimulation and therefore no significant response to lectins (Con-A, E-PHA, PWM) or responsiveness in mixed lymphocyte culture (MLC), whereas mononuclear cells from rib marrow had a low unstimulated background activity and could be stimulated by E-PHA and allogeneic cells in MLC. Bone marrow cells obtained from aspirates and ribs were also studied as to their ability to mediate in vitro cellular cytotoxicity. Antibody-dependent cellular cytotoxicity (ADCC) and lectin-induced cytotoxicity (LICC) were observed using marrow mononuclear cells from both sources when red cells were employed as targets. In contrast, when cell line target cells were employed, rib mononuclear cells were not observed to mediate lysis, whereas mononuclear cells from aspirates did lyse these targets. The cytotoxicity observed with aspirates was therefore most likely due to peripheral blood contamination. In cell-mediated lympholysis (CML), rib mononuclear cells were not stimulated to kill allogeneic lymphocytes, while aspirated cells lysed allogeneic lymphocytes about 50% as well as did peripheral blood mononuclear cells (PBC). Again, a reflection of peripheral blood contamination. We therefore conclude that pure bone marrow from humans, as assessed by studies using curettaged marrow from ribs, contains mononuclear cells capable of responding blastogenically to E-PHA and to cell surface alloantigens, as well as cells able to mediate LICC and ADCC against RBC, but does not include a population of cells able to lyse cell line targets in ADCC, spontaneous cell-mediated cytotoxicity (SCMC), or CML. These results indicate that the clinical evaluation, experimental study, and therapeutic use of bone marrow must be undertaken with the understanding that significant contamination with peripheral blood can occur when aspirates are used and that curettaged or biopsy material be used when true bone marrow is desired or needed.

A PRECISE understanding of the functional capabilities of pure normal human marrow cells is of critical importance for the study of a variety of disease states, including aplastic anemia, leukemia, and the immunodeficiency diseases. Furthermore, with the increasing use of bone marrow transplantation as a mode of therapy for several disease entities, a better delineation of the exact types of cells being transplanted becomes necessary. Unfortunately, lymphoid cells in human bone marrow remain incompletely characterized despite their role as precursor, effector, and regulatory cells of the immune response. This is in part due to the inability to obtain pure marrow specimens and because only a few studies have been done in areas such as the examination of cytotoxic capabilities. Additionally, in areas such as mitogenesis, the results obtained by different investigators have been contradictory. The most likely explanation for previously obtained contradictory results is the inability to obtain pure bone marrow specimens, in that the majority of experiments done to date have utilized conventional marrow aspirates. Varying degrees of peripheral blood contamination in aspirated samples may account for previous contradictory results. Moreover, it has not been possible to arbitrarily correct results of functional assays, since the precise contribution of peripheral blood cells is unknown. Because marrow aspirates are employed in bone marrow transplantation, clinical assessment of disease states, and experimental studies, we have compared the functional capabilities of marrow cells isolated from standard iliac crest aspirates with pure bone marrow obtained by curettage of human ribs. In this manner the contribution of peripheral blood cells in aspirates to apparent immune function could be ascertained by comparison with functional activities obtained from pure rib marrow.

In our present studies comparing aspirates from the iliac crest and curettaged specimens from ribs, we have examined (1) the ability of cells to multiply and divide in response to mitogenic lectins as a function of cell proliferative capacity, (2) the ability of cells to proliferate in response to cell surface antigens as a measure of specific recognition capabilities, and (3) the ability of cells to kill targets (cell-mediated cytotoxicity) as a measure of the effector limb of the immune response. For the evaluation of cell-mediated cytotoxic capabilities, we have employed four systems.

From the Howard Hughes Medical Institute Laboratory and the Division of Gastroenterology, Department of Medicine, Washington University School of Medicine, St. Louis, Mo. Supported in part by USPHS Grants AM21474-01 and AI15322-01. Submitted September 5, 1979; accepted December 17, 1979. Address reprint requests to Charles D. Alley, Ph.D., Division of Gastroenterology, Box 8124, Washington University School of Medicine, 660 S. Euclid, St. Louis, Mo. 63110.

© 1980 by Grune & Stratton, Inc.
0006-4971/80/5504-0021 $01.00/0
First, in direct cell-mediated lympholysis (CML), T lymphocytes become sensitized to allogeneic lymphocytes so that the foreign lymphocytes can be specifically recognized and killed. Second, in antibody-dependent cellular cytotoxicity (ADCC), Fc receptor-bearing lymphocytes kill antibody-coated target cells in a complement-free system. Third, in spontaneous cell-mediated cytotoxicity (SMC), Fc receptor-bearing lymphocytes spontaneously kill target cell lines without prior sensitization. Fourth, in lectin-induced cellular cytotoxicity (LICC), effector cells of any nature are induced by the nonmitogenic lectin wheat germ agglutinin to kill human red blood cells.

In the studies presented below, we have found that pure human bone marrow cells have a set of functional capabilities differing markedly from human peripheral blood lymphocytes. This unique set of functional capabilities is consistent with bone marrow being a site for stem cell and precursor cell development. Furthermore, bone marrow cells obtained by conventional aspiration techniques do not accurately demonstrate the unique functional capabilities of human marrow cells because they are contaminated to a significant degree with circulating mature mononuclear cells. We conclude that the use of aspirates in bone marrow transplantation, study of disease states, and investigation of normal marrow function should be replaced by the use of bone marrow biopsies or curettaged specimens if the results might be influenced by the presence of mature or previously sensitized peripheral blood mononuclear cells (PBC).

**MATERIALS AND METHODS**

**Isolation of Bone Marrow Mononuclear Cells**

The first 3-5 ml obtained from two sites on the posterior iliac crests were pooled for each individual. Aspirates were acquired from 10 normal donors and 7 tumor patients without bone marrow involvement. The individuals were 13-56 yr old and were not on chemotherapy.

Ribs were obtained at surgery from 13 patients undergoing thoracotomy. The patients ranged in age from 16 to 73 yr and none had received chemotherapy or irradiation. The ribs were split and the marrow was scraped into 50 ml of Hanks' balanced salt solution (HBSS), without Ca$^{++}$ or Mg$^{++}$ plus 2.5 mM EDTA (Fisher Scientific Co., Fair Lawn, N.J.) and 500 U sodium heparin (Upjohn, Kalamazoo, Mich.).

The marrow cells were separated and isolated using a discontinuous Ficoll-Hypaque gradient technique as described by Alley and MacDermott. In brief, the EDTA-heparinized marrow was vortexed and then allowed to settle for 10 min at room temperature. The cells in the supernatants were washed twice with HBSS plus EDTA, the red cells lysed by hypotonic lysis in distilled water and the marrow cells allowed to recover in isotonic media 199 (Microbiological Associates, Bethesda, Md.) plus 20% heat-inactivated fetal calf serum (FCS) (GIBCO, Grand Island, N.Y.). The cells to be separated were resuspended at not more than 1.5 x 10$^6$ cells/ml in a solution composed of 6% Ficol (Sigma Chemical Co., St. Louis, Mo.), 6% Hypaque (diatrizoate sodium, Sterling Organics, New York, N.Y.) in 0.4 x HBSS plus 2.5 mM EDTA and 0.1 ml/ml Hepes buffer (GIBCO, Grand Island, N.Y.), having a specific gravity of 1.050 g/cc, a pH of 7.2, and an osmolality of 298 mosmole. Two milliliters of this suspension was layered over a discontinuous gradient composed of 2 ml of each of 4 Ficoll-Hypaque solutions. Each solution contained 0.1 ml/ml Hepes buffer, had a pH of 7.2, and an osmolality of 298 mosmole. Solution I was composed of 8% Hypaque, 8.5% Ficol in 0.1 X HBSS and had a specific gravity of 1.060 g/cc. Solution II had a specific gravity of 1.070 g/cc and was made of 9% Hypaque and 8.9% Ficol in sterile distilled water. Ten percent Hypaque and 6.4% Ficol in sterile distilled water were used to construct Solution III with a specific gravity of 1.077 g/cc. Solution IV consisted of 6.3% Hypaque and 23% Ficol dissolved in 0.6 x HBSS and had a specific gravity of 1.085 g/cc. The gradients were centrifuged at 400 g for 40 min at room temperature. The cell populations found at each gradient interface were harvested, and cells from the corresponding interfaces of each gradient were pooled. The cells were washed twice in HBSS plus EDTA followed by 2 additional washes in media 199 plus 10% FCS.

Separation of aspirated or rib marrow over the Ficoll-Hypaque discontinuous gradients resulted in approximately 5%-10% of the recovered cells being harvested from the interface above solution I; 30%-40% from the interface between solutions II and III; and 30%-50% from the interface between solutions III and IV. The pellet was composed of non-nucleated RBC with negligible numbers of nucleated cells. The average total cell recovery from the gradients was 54.0% ± 4.4% with a range from 36.0% to 82.0% and a viability of greater than 95% by trypan blue exclusion.

When examined microscopically, the cellular composition of each layer was different, with the mononuclear cells predominantly found in layer 2 (interface between solutions I and II, hereafter referred to as the mononuclear fraction) and granulocytes predominating in layer 4 (interface between solutions III and IV, hereafter referred to as the granulocytic fraction). The mononuclear fraction separated from aspirated human bone marrow was approximately 70% mononuclear cells and 20% blast cells, while the granulocytic fraction was 80% granulocytes and only 8% blasts. On the other hand, with rib marrow, we observed approximately 50% mononuclear cells, 20% granulocytes, and 15% blast cells in the mononuclear cells, and 70% granulocytes and 9% blasts in the granulocytic fraction. Of particular interest was the difference in the proportion of nucleated RBC observed in the mononuclear fraction of rib and aspirated marrow, in that rib marrow contained 3 times as many nucleated RBC as did the corresponding fraction of aspirated marrow (15% versus 5%), thus indicating the presence of immature erythroid elements. In analyzing the cell types present in the mononuclear fraction, we found a higher proportion of mature forms in the marrow mononuclear cell fraction from aspirate than rib, particularly with regard to T cells (9.1% versus 3.6%), B cells (25.9% versus 18.7%), and macrophages (8.0% versus 5.4%). By calculation, the percentage of null cells (non-T, non-B, nonmacrophage) was higher in the marrow mononuclear cells isolated from rib (79.5%) than those from aspirates (45.0%). However, the proportion of Fc receptor-bearing cells was essentially the same in both rib and aspirate specimens (14.3% and 13.0%, respectively). The increase in SIg-positive cells found in the marrow preparations over that found in peripheral blood was due to large blast-like cells. These cells were greater than 13 μm in diameter and brightly fluorescent. Cells of this description were predominant in rib mononuclear fractions, present to a lesser extent in aspirated mononuclear fractions, and were not seen in peripheral blood.

**Blastogenic Response of Lymphoid Cells**

Peripheral blood mononuclear cells and bone marrow cells were assayed simultaneously for their response to several mitogens. The
proliferation response was investigated using a modification of standard technique as previously described.\(^1\) Briefly, \(10^5\) cells were pipetted into flat-bottom wells of microtiter plates (Costar, Cambridge, Mass.) into which 0.1 ml of the mitogens had been pipetted in triplicate. The mitogens employed were Con-A, 10 \(\mu g/ml\) (A grade, Calbiochem, LaJolla, Calif.), erythroagglutinating fraction of phytohemagglutinin (E-PHA), 10 \(\mu g/ml\) (PHA-P, Difco, Detroit, Mich. separated as described by Weber, Nordman, and Grasbeck\(^9\)) and pokeweed mitogen (PWM), 1:100 dilution. The media plus gentamicin (Schering Corp., Kenilworth, N.J.), 2% 200 mM L-glutamine, and 1% 1 M Hepes buffer (GIBCO, Grand Island, N.Y.) were added to the cells and mitogens in a total of 0.2 ml of RPMI 1640 plus 20\% heat-inactivated pooled normal human male sera were plated in triplicate in round-bottom well microtiter plates (Titertek, Linbro Scientific, Inc., Hamden, Conn.). Additional wells were plated with responding or stimulating cells plus PHA. On day 5, the cultures were pulsed with 0.05 ml \(\text{H}^3\text{H}\) thymidine (specific activity 46 Ci/m mole, New England Nuclear, Boston, Mass.) on the second day. After an additional 18 hr in culture, the cells were harvested, radioactivity was counted, and stimulation indices were calculated as the cpm experimental divided by the cpm background.

Stimulation of marrow and peripheral blood mononuclear cells by allogeneic mononuclear cells was studied by modifications of the standard one-way MLC.\(^2\) In brief, \(5 \times 10^5\) responding cells and \(5 \times 10^5\) irradiated (4000 R) stimulating PBC in a total of 0.2 ml of RPMI 1640 plus 20\% heat-inactivated pooled normal human male sera were plated in triplicate in round-bottom well microtiter plates (Titertek, Linbro Scientific, Inc., Hamden, Conn.). Additional cells were plated with responding or stimulating cells plus PHA. On day 5, the cultures were pulsed with 0.05 ml \(\text{H}^3\text{H}\) thymidine and incubated for an additional 18 hr. The cultures were harvested as above.

**Cytotoxicity Assays**

RBC targets (chicken or human) were prepared by drawing 2 ml of whole blood on the day on which the assays were performed. The blood was washed once in HBSS without Ca\(^{++}\) and Mg\(^{++}\) and twice in media 199 (Microbiological Associates, Bethesda, Md.) with 2% L-glutamine, 50% 1 M NaCl, 1% 1 M Hepes buffer, and 10\% heat inactivated FCS (GIBCO, Grand Island, N.Y.) plus 50 \(\mu g/ml\) gentamicin. The pellets were resuspended in 0.05 ml of sodium chromate (\(\text{Cr}^{51}\), 250 \(\mu C\); specific activity 300 mCi/mg, New England Nuclear, Boston, Mass.) and incubated for 2 hr at 37\(^\circ\)C with resuspension every 15 min. The labeled RBC were washed 3 times in media 199, counted, and diluted to a concentration of 2 \(\times 10^6/ml\). Nucleated cell line targets (Chang liver cells and K562 lymphoid cells) were harvested from tissue culture without the use of trypsin. The cells were resuspended in 0.05 ml \(\text{Cr}^{51}\) and incubated for 1 hr at 37\(^\circ\)C with resuspension every 15 min. The labeled cells were washed 3 times, counted, and diluted to 5 \(\times 10^5\) cells/ml. PBC from the two individuals used as stimulators were used as targets in the CML. The targets were cultured at a concentration of 10\(^4\)/ml in RPMI 1640 (Microbiological Associates, Bethesda, Md.) with 2% L-glutamine, 5% gentamicin, 1% 1 M Hepes buffer plus 20\% heat-inactivated pooled normal human male serum. On day 6 of culture, the cells were harvested, centrifuged through Ficoll-Hypaque,\(^10\) and labeled with \(\text{Cr}^{51}\) as were the nucleated cell line targets above.

To obtain antiserum for the assays, rabbits were injected intravenously (ear vein) with 0.5 ml saline containing target cells (5 \(\times 10^6\) chicken RBC or 4 \(\times 10^6\) Chang liver cells), 5 times at weekly intervals, and then bled.

ADCC employing chicken RBC and Chang cell line cells as targets, SCMC employing K562 cell line cells and LICC using human RBC were performed in round-bottom well microtiter plates (Titertek, Linbro Scientific, Inc., Hamden, Conn.). A volume of 0.05 ml of the labeled target cell suspension (5000 Chang liver cells or K562 lymphoid cells and 10,000 chicken or human RBC) was pipetted into each well. For the ADCC assays, the appropriate antiserum was thawed and diluted with media 199 to a concentration of 1/100 for rabbit anti-Chang liver cell antiserum and 1/333 for rabbit anti-chicken RBC antiserum. In the LICC, wheat germ agglutinin (WGA, Lot WG39, Miles Labs Inc., Elkhart, Ind.), at a stock concentration of 2.7 mg/ml HBSS, was thawed and diluted with media 199 to a concentration of 1/180. A volume of 0.05 ml of antiserum or lectin was plated in triplicate for each effector cell to target cell ratio studied. An additional 3 wells for each ratio received 0.05 ml of media 199 in lieu of antiserum or lectin.

The SCMC, Chang-ADCC, and WGA-LICC assays were incubated for 18 hr at 37\(^\circ\)C in a 5\% CO\(_2\) moist air atmosphere before harvesting. The chick-ADCC assay was centrifuged at 50 g for 5 min before incubation for 4 hr at 37\(^\circ\)C in a 5\% CO\(_2\) moist air atmosphere. All assays were harvested by the Titertek supernatant collecting system (Flow Laboratories, Rockville, Md.). The supernatant-containing filters were counted on a Beckmann Gamma 400 counter (Beckman Instruments, Inc., Irvine, Calif.). The triplicate containing detergent (Triton X-100) was considered the maximal release and that containing the media plus target cells the spontaneous release. The percent cytotoxicity was determined by the formula:

\[
\text{Percent Cytotoxicity} = \frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Maximal cpm} - \text{Spontaneous cpm}} \times 100
\]

Cytotoxicity activity of bone marrow cells and PBC after stimulation by allogeneic PBC was studied by a modification of the CML technique of Hirshberg and coworkers.\(^11\) In brief, \(5 \times 10^5\) effector marrow cells or PBC and \(5 \times 10^4\) irradiated (4000 R) stimulating PBC in a total of 0.2 ml RPMI 1640 plus 20\% heat-inactivated pooled normal human male sera were plated in triplicate in round-bottom well microtiter plates. On day 6, the cultures were resuspended by repeated pipetting and 10\(^4\) \(\text{Cr}^{51}\)-labeled allogeneic PBC target cells were added (effector cell to target cell ratio of 5:1). The cultures were incubated an additional 5 hr at 37\(^\circ\)C in a 5\% CO\(_2\) in air atmosphere. Harvesting and counting of the cultures were performed as reported above for the cytotoxicity assays. Cytotoxic activity is reported as the cytotoxic index (CI) for comparative purposes. CI was calculated according to the following formula:

\[
\text{CI} = \frac{\% \text{Cytotoxicity exp.} \times 100}{\% \text{Cytotoxicity exp.} \times 100 - \% \text{Cytotoxicity control} \times 100}
\]

where the percent cytotoxicity was calculated using the formula noted above, controls were the allogeneic PBC effector:target CML, and the background was the percent \(\text{Cr}^{51}\) release observed in wells containing target cells and media only.

**Statistical Analysis**

The statistical significance of differences was determined using the Students' t test.

**Macrophage Depletion**

Adherent cells were depleted from the mononuclear cell populations isolated from 3 ribs by passage over a G-10 Sephadex (Sigma Chemical Co., St. Louis, Mo.) column. The nonspecific esterase technique for monocytes and macrophages was performed on the pre- and postcolumn populations.\(^12\)
RESULTS

Stimulation by Mitogenic Lectins and Cell Surface Antigens

We first examined responsiveness to the mitogenic lectins Con-A, E-PHA, and PWM and to allogeneic cell surface antigens in mixed lymphocyte culture (MLC) in order to assess cell recognition capabilities. Figure 1 shows the stimulation indices due to lectin stimulation for aspirate and rib mononuclear cells as well as peripheral blood cells. When the blastogenic response of rib marrow, aspirated marrow, and peripheral blood mononuclear cells were compared, different results were observed depending on the source of the cells. Mononuclear cells from aspirates displayed an exceedingly high degree of background activity (5171 ± 890 cpm), which rendered any stimulation in terms of stimulation index insignificant. In direct contrast, unstimulated background activity of the rib mononuclear cells was significantly lower (204 ± 59 cpm; p < 0.001). When the average stimulation indices from 5 aspirates were calculated (Fig. 1), there was essentially no observable stimulation of the aspirate mononuclear cells by mitogenic lectins due to the high background activity. On the other hand, the low background activity present in curettaged rib marrow allowed significant stimulation by E-PHA to be observed (Fig. 1). Investigation of the stimulation of marrow mononuclear cells by allogeneic cells in a one-way MLC demonstrated that the background activity for mononuclear cells from aspirated marrow was again very high, rendering the stimulation of these cells insignificant. However, when rib mononuclear cells were studied, the low background activity allowed significant stimulation by allogeneic cells to be observed (Fig. 2). The stimulation indices calculated for rib mononuclear cells stimulated by irradiated allogeneic peripheral blood mononuclear cells averaged 8.2 ± 2.0 and ranged from 3.2 to 14.9 (Fig. 2). Under the MLC culture conditions, the rib mononuclear cells were again stimulated very well by E-PHA (Fig. 2).

Cytotoxic Capabilities Against Red Cell and Cell Line Targets

Human red blood cells were lysed by bone marrow mononuclear cells separated from both aspirates and ribs, when induced by the nonmitogenic lectin wheat germ agglutinin (Table I). Macrophage-depleted bone marrow mononuclear cells also performed at the same level (data not shown). Mononuclear cells from both aspirates and ribs also demonstrated excellent cytotoxic capabilities toward chicken red blood cells in the presence of anti-chick RBC antibody (Table I). Macrophage depletion by the G-10 Sephadex adherence column method had no effect on the ability of bone marrow mononuclear cells from 3 ribs to lyse chick RBC (data not shown).

Cells capable of mediating either SCMC or ADCC of Chang cell line nucleated target cells were not detected in marrow from ribs (Table 1). Furthermore, neither unseparated bone marrow cells nor granulocytic cells from ribs displayed any activity in either system employing Chang liver cells as targets (data not shown). On the other hand, consistent cytotoxic
activity was noted by mononuclear cells separated from aspirates. This activity was best observed at an effector cell to target cell ratio of 50:1; however, the aspirated mononuclear cells were significantly poorer (p < 0.001) in mediating cytotoxicity in these systems than were peripheral blood cells, but were significantly (p < 0.01) better than rib mononuclear cells.

The same pattern of cytotoxicity by aspirate or rib mononuclear cells seen in the Chang ADCC and SCMC assays was observed in the K562 SCMC assay (Table 1). There was no cytotoxic activity noted using rib cells at any of the effector cell to target cell ratios employed. On the other hand, mononuclear cells isolated from aspirated marrow exhibited 8%-9% cytotoxicity toward K562 lymphoid cells (Table 1), a level that was significantly higher (p < 0.01) than that observed for rib mononuclear cells, and significantly lower (p < 0.001) than that observed for peripheral blood cells.

Cytotoxic Capabilities in Cell-Mediated Lympholysis

The ability of bone marrow mononuclear cells from ribs and aspirates to specifically lyse allogeneic lymphocytes by which they were stimulated was studied. Control peripheral blood cells from a third donor (CPBC) were observed to kill allogeneic target cells at the same level as the allogeneic control cells (Table 2). Mononuclear cells separated from aspirates specifically lysed allogeneic cells at a level approximately 50% lower than the CPBC (Table 2). Little or no activity was observed when mononuclear cells from ribs were tested (Table 2). All 3 groups were found to be significantly different (p < 0.01) in their activity.

DISCUSSION

In the present study, a number of functional differences between human bone marrow mononuclear cells isolated by aspiration from the iliac crest and human bone marrow mononuclear cells isolated by curettage from ribs were observed. Mononuclear cells from aspirated marrow were noted to have a high spontaneous background activity rendering stimulation by mitogenic lectins or allogenic cells insignificant. On the other hand, mononuclear cells obtained from rib specimens by curettage did not have high spontaneous background activity, and stimulation by E-PHA and allogeneic cell surface antigens in MLC could be observed. When cytotoxic capabilities were examined, mononuclear cells from ribs showed no activity in antibody-dependent cellular cytotoxicity (ADCC) or spontaneous cell-mediated cytotoxicity (SCMC) when cell lines were used as targets, nor did they kill in the cell-mediated lympholysis assay (CML). In contrast, mononuclear cells obtained by aspiration from iliac crest did show an intermediate degree of effector cell capability in the ADCC and SCMC assays with cell lines as targets and in the CML assay. Finally, mononuclear cells from both aspirates and ribs were excellent mediators of ADCC and lectin-induced cellular cytotoxicity (LICC) when red cells were used as targets. The implication of these findings is that human bone marrow mononuclear cells obtained by aspiration are functionally different from mononuclear cells obtained by curettage. This is most likely due to contamination of bone marrow aspirates with peripheral blood, as suggested by previous investiga-

<table>
<thead>
<tr>
<th>Table 1. Cytotoxic Capabilities of Bone Marrow Mononuclear Cells Against Red Cell and Cell Line Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Killer to</td>
</tr>
<tr>
<td>Target Cell Ratio</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>Lateral crest aspirate</td>
</tr>
<tr>
<td>25:1</td>
</tr>
<tr>
<td>Peripheral blood†</td>
</tr>
<tr>
<td>25:1</td>
</tr>
<tr>
<td>Rib curettage</td>
</tr>
<tr>
<td>25:1</td>
</tr>
<tr>
<td>Peripheral blood‡</td>
</tr>
<tr>
<td>25:1</td>
</tr>
</tbody>
</table>

*Mean ± standard error of the mean. All values are for five separate experiments, each of which was done in triplicate. Similar results were obtained at 10:1 killer to target cell ratios.
†Simultaneous controls for experiments using aspirates.
‡Simultaneous controls for experiments using ribs.

<table>
<thead>
<tr>
<th>Table 2. Comparison of Cytotoxic Ability of Bone Marrow Mononuclear Cells from Aspirates and Ribs in CML</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytotoxic Index</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>A PBC Targets</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>C PBC</td>
</tr>
<tr>
<td>Aspirates</td>
</tr>
<tr>
<td>Ribs</td>
</tr>
</tbody>
</table>

*Mean ± SEM for five separate experiments.
The use of bone marrow specimens for therapeutic, diagnostic, or experimental purposes necessitates careful consideration of the source of the cells and degree of contamination with peripheral blood before accurate data can be obtained and reliable decisions can be made.

A number of studies have addressed the question of whether or not human bone marrow cells can respond to mitogenic lectins. Because all of the previous studies employed marrow obtained by aspiration, we feel that some of the contradictory results previously obtained might be due to varying degrees of peripheral blood contamination. Indeed, in our present study, a marked difference between human marrow obtained by aspiration and human marrow obtained from ribs was observed, with the observed observation being that human bone marrow will respond to E-PHA and to allogeneic cell surface antigens in MLC. However, the major difference between aspirate and rib marrow was the markedly elevated background activity in aspirated marrow specimens, which rendered determination of stimulation indices insignificant. While varying degrees of baseline activity might account for the wide range of results noted by previous investigators, the question remains as to why aspirated human bone marrow should have such a markedly elevated background activity while rib marrow does not. One possible explanation is that the contaminating peripheral blood mononuclear cells are being stimulated in vitro by the immature bone marrow cells in an autologous mixed lymphocyte reaction. Another possibility would be that for unknown reasons bone marrow from the iliac crest might have different functional characteristics than bone marrow from ribs. A third possibility is that peripheral blood cells, which circulate in the area of the iliac crest marrow and are aspirated in the iliac crest marrow, are themselves a unique cell type with a high spontaneous activity.

Regardless of the mechanism for the high spontaneous background activity, the ability of pure bone marrow cells isolated by curettage from ribs to respond to E-PHA and to allogeneic cell surface antigens is somewhat surprising in view of the small number of T cells present as assessed by the E-rosetting technique. That is, with only 3% of the cells being T cells, why should they respond blastogenically? The most likely explanation is that the T cells present constitute a unique subpopulation of cells which for some reason are able to respond to allogeneic cell surface antigens. It should be noted that the degree of stimulation is not as great as that found in peripheral blood, and thus, the majority of the blastogenesis may indeed be due to the few mature T cells normally present in pure human marrow. Alternatively, the observed stimulation may be due to maturation of precursor cells present in human bone marrow into functionally mature T cells. The questions that have been raised by the present studies with regard to the reason for the high background activity in aspirated marrow specimens and an explanation of why pure human marrow should be capable of responding to mitogen and antigen stimulation despite the absence of T cells will require further studies in order to answer.

The present study is the first attempt to delineate the cytotoxic effector capabilities of human bone marrow cells using cell lines as targets and also is the first study investigating effector capabilities in the cell-mediated lympholysis (CML) assay. Our results indicate that pure human bone marrow mononuclear cells isolated by curettage from ribs are unable to act as either ADCC or SCMC effector cells against cell line cells and also do not function in the CML assay. We also noted that if human bone marrow mononuclear cells are obtained by aspiration, an intermediate degree of activity is noted in these assays. The most likely explanation for the difference between the results with aspirates and those with ribs is that the activity observed with aspirates is in fact not due to bone marrow cells, but rather due to contamination by peripheral blood. An alternative explanation is that during the sensitization or incubation periods, contaminating blood T cells allow precursor cells to develop into true bone marrow effector cells when aspirates are used, while curettaged marrow is unable to allow its precursor cells to develop into effector cells. We also observed that with red cells as targets, both ADCC and LICC could be achieved by bone marrow cells, which is in agreement with the previous study of Fauci et al. using red cells as targets. The studies with red cells as targets are of importance in that they demonstrate that the bone marrow cells were functionally active and not nonspecifically destroyed by the isolation procedures. In addition, there is an interesting dichotomy between ability to function as effector cells of ADCC with red cells as targets and the inability to function as effector cells of ADCC with cell lines as targets. It may be that there are multiple subgroups of Fc-bearing cells that act as effector cells of ADCC in the peripheral blood and that bone marrow cells have a unique group of Fc receptor-bearing cells that function with one type of cell as a target but not with another type of cell as a target. Alternatively, there may be other receptors of importance in determining whether or not an Fc receptor-bearing cell can interact with a target, and these other receptors may be missing in the bone marrow compartment.

While the ADCC and SCMC assays evaluate the
functional capabilities of Fc receptor-bearing cells, the CML assay evaluates the functional capabilities of T cells as effector cells. In our hands, bone marrow cells from aspirates were 50% as effective as peripheral blood cells, while bone marrow cells from ribs were not effectors of CML. One of the areas in which this observation may be of great importance is in bone marrow transplantation; that is, in allogeneic bone marrow transplantation. If the bone marrow aspirates contain significant numbers of allogeneic T cells capable of mounting a CML reaction, these cells may also be able to participate in the graft-versus-host reactions that are so commonly observed after this procedure. On the other hand, marrow cells obtained by curettage do not appear to contain mature T cells with significant cytotoxic effector capabilities. One intriguing question raised by this last observation is what signals are necessary for the development of cytotoxic effector cells. In this regard, it would be of interest to examine inducing agents such as thymosin, interferon, and growth factors for their ability to promote maturation of cytotoxic effector cells.

Our source of uncontaminated human bone marrow in the present study was ribs removed surgically at thoracotomy, and therefore, adds to the evidence that ribs will offer a useful source of pure marrow cells. Recently, deGast and Platts-Mills have employed sections of ribs removed during thoracotomy and found that the isolated marrow cells do not synthesize or secrete antibodies. In addition, Ross and coworkers have utilized marrow from human ribs to study the development of granulocytes. Therefore, we feel that further studies employing ribs will allow a more clear evaluation of the true functional and developmental capabilities of human bone marrow cells.

In the present study we have found that bone marrow mononuclear cells are a unique cell type, distinctly different from peripheral blood cells in that bone marrow mononuclear cells do not respond to the mitogenic lectins concanavalin A or pokeweed mitogen and do not mediate cytotoxicity against either cell line targets or lymphocytes in the CML assay. In contrast, peripheral blood cells are excellent mediators in the cytotoxicity assays and respond well to the mitogenic lectins. The most likely explanation for these results is that bone marrow mononuclear cells are predominantly immature populations of stem cells and precursor cells in which the effector functions, which represent mature lymphocyte activities, are not yet present. This would be consistent with the bone marrow compartment as a site for cells in a unique stage of their development. It also raises the point as to what studies of pure human marrow are the most relevant for the understanding of this unique organ. We feel that the study of stem cell development into precursor and effector cells through the use of colonies in agar or in liquid culture constitute an area of great interest for future investigations of pure human bone marrow. Furthermore, studies with regard to the development of stem cells or precursor cells into cytotoxic effector cells also would be an area of great importance. In view of the evidence that pure bone marrow lymphocytes do not synthesize antibody (preliminary studies from our laboratory), another area of great importance would be systems in which the ability of stem cells develop into antibody-synthesizing cells can be examined. With human marrow, in all of the above potential areas of investigation, we would recommend that curettaged marrow specimens be used to prevent inaccurate results due to contaminating peripheral blood cells.

ACKNOWLEDGMENT

The authors gratefully acknowledge Drs. Charles Roper and Martin Bergmann for furnishing the ribs and Drs. Gordon Phillips and Geoffrey Herzig for providing the aspirates. The technical assistance of Michael A. McGraw and Jan Bragdon and the secretarial expertise of Catherine Camp and Pam Helms are also greatly appreciated.

REFERENCES

9. Weber T, Nordman CT, Grasbeck R: Separation of lymphocyte stimulating and agglutinating activities in phytohemagglutinin


Investigation of the blastogenic and cytotoxic capabilities of human bone marrow: differences between aspirate and rib marrow

CD Alley and RP MacDermott