Characterization of the different lymphocyte populations in normal human bone marrow (BM) was attempted and compared to that in the peripheral blood (PB). B cells comprised 34% ± 11% of lymphocytes in BM and 23% ± 9% in PB. The majority of B cells carried IgM in BM and IgG in the PB. In the BM, cells carrying complement or Fc receptors were fewer than cells carrying Ig, but in the PB they were equal. T cells comprised 6% ± 4% of lymphocytes in the BM and 62% ± 7% in the PB. The majority of BM lymphocytes did not have B or T cell markers; these probably included B and T cell precursors. BM lymphocytes carrying surface Ig increased in a 7-day culture, whereas those of the PB decreased. Pokeweed mitogen induced Ig synthesis in B cells of PB but not those of BM. BM-T cells were more efficient than PB-T cells in inhibiting Ig synthesis of PB-B cells. These results indicate that the BM compartment contains immature B cells that are capable of partial differentiation and maturation in vitro. BM-B lymphocytes are probably not involved in the effector phase of the immune response since they are unable to synthesize Ig and because they carry few receptors for complement or Fc. BM-T lymphocytes are very few and have suppressor capability and therefore may play an essential role in regulation of Ig synthesis by B cells.

THE BONE MARROW compartment is probably the main source of stem cells and bone marrow-dependent (B) cells in adult life. We have previously demonstrated that the bone marrow is also a unique compartment in that B cells carry predominantly IgM receptors. In postnatal life, human marrow may function as the bursa equivalent in man, but this has not been completely proven as yet.

In this study, we have attempted to clarify further the status of the B and T cell subpopulations and precursors among bone marrow lymphocytes and their ability to differentiate and mature.

MATERIAL AND METHODS

Subjects and Sample Collection
Bone marrow aspirates were obtained from 17 donors who were undergoing evaluation for mild anemia. The donors' ages were 26-61, and they did not present with apparent immunologic diseases. None of the patients was receiving steroids or cytotoxic drugs. Bone marrow aspirates, 1.0-2.0 ml, were collected in heparin, and only those with marrow particles were used. At the time of bone marrow collection, 20 ml of peripheral blood was also drawn into heparin. Total nucleated cell counts and viability studies using the trypan blue exclusion test were done.
Preparation of Cell Suspensions

Bone marrow mononuclear rich cell suspensions were collected by the linear sucrose density gradient centrifugation technique described by us earlier.2 The standard Ficoll-Hypaque technique8 was not adequate for bone marrow fractionation. The mononuclear-rich upper portion of the sucrose gradient was collected and washed three times in Medium 199 (Microbiological Associates, Bethesda, Md.). A smear was made of the final suspension and stained with Wright-Giemsa stain. The bone marrow suspensions after fractionation on the sucrose gradient contained 32-46 x 10⁶ cells of which 91%-97% were small mononuclear cells and 87%-94% were viable. This procedure yielded 12%–19% of the nucleated cells and a majority of the small mononuclear cells present in the unfractonated bone marrow.

Peripheral blood lymphocytes were separated by the standard Ficoll-Hypaque technique.8 The final mononuclear-rich cell suspension harvested from 12 to 20 ml blood contained 9-14 x 10⁶ cells, of which 93%-97% were small mononuclear cells and 91%-98% were viable. This procedure yielded 17%–29% of the nucleated cells and 85%-96% of the mononuclear cells in the unfractonated population.

Studies of B Cells

B cells were enumerated by the three known B cell markers. (1) Surface membrane immunoglobulins were studied using fluorescinated goat antihuman IgG, M, A, k, l, and whole Ig antiserum from commercial sources (Behring Diagnostics, Woodbury, N.Y.). Anti-IgD was not used. Antisera were tested for specificity by immuno-electrophoresis against normal serum. We have not used F(ab)₂ fragments of the antisera. The technique of staining B cells was described earlier.9 (2) Complement receptors were studied by the EAC rosette assay as described earlier.9 In all the experiments, we used fresh mouse serum to coat the EA. (3) The Fc receptors were tested by binding of cells to fluorescinated aggregated human gamma globulin.10

The capacity of B cells to synthesize Ig was tested by incubating 1 x 10⁶ cells/tube in 1 ml Medium 199-10% fetal calf serum (FCS) (Microbiological Associates). Pokeweed mitogen (PWM) (Gibco, Grand Island, N.Y.) 10 μg/tube was added to the experimental tubes.11 The tubes were then placed in a humid atmosphere of 5% CO₂ in air at 37°C for 7 days. Following incubation the cells were washed three times in phosphate-buffered saline (PBS), pH 7.4, and 0.2 x 10⁶ cell aliquots were placed in cytocentrifuge chambers (Shandon Southern Instruments Co., Sewickley, Pa.) and spun at 200 rpm at room temperature. Slides were then air dried and placed in ice-cold 95% ethanol-5% acetic acid for 20 min. Cells were then washed with fluorescinated goat antihuman Ig antiserum (Behring Diagnostics) for 30 min at room temperature in a humid chamber. Slides were washed three times with PBS and mounted with glycerine-PBS and kept at 4°C. The slides were read with a fluorescent microscope using an HBO 200 mercury arc, BG 12 excitation filter, KP 490 and 510 barrier filter. Cells with bright cytoplasmic fluorescence were counted as positive cells. T cells were enumerated using the E rosette assay12 and monocytes by the EA rosette assay.13

Preparation of Enriched T and B Cell Suspensions

Bone marrow or peripheral blood lymphocytes were allowed to form E rosettes.12 Following 1-hr incubation at 4°C, the rosettes were suspended gently. The suspension was then diluted with chilled Medium 199 in a ratio of two volumes of the rosette suspension to one volume of the medium. Five parts of the diluted rosette suspension were then layered on three parts Hypaque-Ficoll gradient. The tubes were centrifuged at 700 g for 35 min at 10°C. Cells in the upper layer of the gradient were found to be predominantly B cells (90% ± 9%), in peripheral blood and 46% ± 11%, in bone marrow. Cells in the pelot were predominantly T cells (91% ± 8%) in peripheral blood and 53% ± 19% in bone marrow. Sheep red cells in the pelot were lysed by hypotonic shock, and both the B and T cells were washed with Medium 199 before use.

Testing for Suppressor T Cell Function

One million T and B cells were cocultured in 1 ml Medium 199-10% FCS in the presence of 10 μg PWM in a humid atmosphere of 5% CO₂ in air. Controls were T cells or B cells cultured alone.
Table 1. Distribution of Various Mononuclear Subpopulations in Human Bone Marrow and Peripheral Blood

<table>
<thead>
<tr>
<th>Cell Source (no. patients)</th>
<th>B Cells</th>
<th>T Cells</th>
<th>Monocytes</th>
<th>Null Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SmIg*</td>
<td>Agg GG</td>
<td>EAC Rosettes</td>
<td>EA Rosettes</td>
</tr>
<tr>
<td>BM (17)</td>
<td>34 ± 11</td>
<td>11 ± 3</td>
<td>13 ± 4†</td>
<td>6 ± 4‡</td>
</tr>
<tr>
<td>PB (19)</td>
<td>27 ± 9</td>
<td>24 ± 7</td>
<td>23 ± 5</td>
<td>61 ± 7</td>
</tr>
</tbody>
</table>

Amounts shown are percentages ±SD.
*Cells stained for surface-membrane Ig at 4°C by FITC anti-human Ig antiserum.
†Aggregated human gamma globulin.
‡Percentage of null cells is calculated by subtracting from 100 the figure obtained by adding percentage E rosettes to percentage of cells with SmIg.
§p < 0.01 when compared to BM-B cells with SmIg.
pp < 0.001 when compared to PB cells of the same type.
||p < 0.01 when compared to BM cells of the same type.

In all the experimental cultures, T cells from either blood or bone marrow were cocultured with B cells from blood only. B cells of bone marrow were not cocultured with T cells because of their lack of synthesis of Ig. At the end of the 7 day culture the cells were washed twice in Medium 199 and processed for cytoplasmic fluorescence as described above. The percentage of fluorescing cells (B cells) was corrected by the percentage of negative T cells present in the culture.

RESULTS

Table 1 shows the distribution of the various mononuclear subpopulations in bone marrow (BM) and peripheral blood (PB). B cells were quantitated by the three different membrane markers. Though surface membrane immunoglobulin (SmIg) carrying B cells were increased in BM, the differences between BM and PB were not statistically significant. B cells carrying receptors for the aggregated human gammaglobulin (Agg GG) or for complement (EAC) were significantly fewer in BM when compared to BM-B cells carrying SmIg or to PB-B cells carrying the complement or Fc receptors. T cells were very few in BM, compared to those in PB (p < 0.001). Monocytes as detected by the human IgG receptor (EA rosettes) were present in both BM and PB. The percentage of cells lacking both the B (SmIg) and T (E rosette) markers, called "null" cells, constituted the majority (60% ± 13%) of the BM lymphocytes and were significantly greater than null cells in PB (p < 0.001).

The various SmIg classes of B cells are listed in Table 2. The majority of B cells in BM carried IgM, while those in PB were IgG. IgG and IgA carrying cells were significantly fewer in BM than in PB. The ratio of B cells carrying κ chains to those carrying λ chains was 1.2:1 in BM and 1.9:1 in PB.

Table 2. B Cell Surface-Membrane Ig Classes in Human Bone Marrow and Peripheral Blood

<table>
<thead>
<tr>
<th>Cell Source (no. exps.)</th>
<th>Anti-Whole Ig</th>
<th>Anti-IgM</th>
<th>Anti-IgG</th>
<th>Anti-IgA</th>
<th>Anti-κ</th>
<th>Anti-λ</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM (9)</td>
<td>34 ± 10</td>
<td>21 ± 7</td>
<td>9 ± 5</td>
<td>11 ± 3</td>
<td>21 ± 7</td>
<td>17 ± 4</td>
</tr>
<tr>
<td>PB (12)</td>
<td>25 ± 9</td>
<td>6 ± 3</td>
<td>17 ± 5</td>
<td>7 ± 3</td>
<td>15 ± 6</td>
<td>8 ± 5</td>
</tr>
</tbody>
</table>

*FITC antihuman globulin heavy chain or light chain specific. F(ab)_2 fragments were not used.
†Antiserum raised against all Ig classes.
‡p < 0.01 when compared to PB cells carrying same Ig class.
Table 3. B Cell Precursors and Subpopulations in Human Bone Marrow and Peripheral Blood

<table>
<thead>
<tr>
<th>Cell Source (no. expr.)</th>
<th>Surface-Membrane Ig* on Day</th>
<th>Cytoplasmic Ig** on Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>BM</td>
<td>31</td>
<td>27</td>
</tr>
<tr>
<td>(5)</td>
<td>± 7</td>
<td>± 9</td>
</tr>
<tr>
<td>PB</td>
<td>25</td>
<td>17</td>
</tr>
<tr>
<td>(7)</td>
<td>± 5</td>
<td>± 7</td>
</tr>
</tbody>
</table>

*Cells stained at 4°C by FITC antihuman Ig antiserum. Mean percents ± SD of positive cells are shown.

†Cells cultured with 10 μg PWM in 10% FCS and then stained by FITC antihuman Ig antiserum. Mean percents ± SD of positive cells are shown.

p < 0.01 when compared to PB cells of same day.

The in vitro maturation and differentiation of B cells was tested by examining SmIg and Ig synthesis (cytoplasmic Ig) at various intervals following incubation in vitro. Because of the poor viability of the cultured cells after the seventh day, we examined the cells up to the seventh day only. Table 3 demonstrates that BM-B cells carrying SmIg were increased in vitro, whereas PB-B cells decreased. The differences between the two cell populations were significant on days 2 and 7. Though there was an increase in BM-B cells carrying SmIg, from 31% on day 0 to 53% on day 7, cells synthesizing Ig as detected by the cytoplasmic immunofluorescent technique were only 5% on day 7. At the onset of culture, 25% of PB cells carried SmIg and were considered to be B cells. This B cell subpopulation significantly decreased to 3% after 7 days in culture. The PB-B cell subpopulation with Ig synthetic capability, when cultured under similar conditions, showed a significant increase from 0% at the onset of culture to 17% on day 7.

The increase or decrease of the various B cell subpopulations in both BM or PB was not due to a selective elimination of nonlymphoid cells during the 7-day culture. At day 0, >90% of BM cells and >87% of PB cells were viable. (See Materials and Methods.) At day 0 we cultured 1 × 10⁶ cells, and at day 7 total cell recovery was 0.62 ± 0.21 × 10⁶ (mean ± SD) in BM cultures and 0.51 ± 0.17 in PB cultures. Viable cells on day 7 were 64% ± 21% in BM cultures and 74% ± 11% in PB cultures. In both BM and PB cultures, cells recovered on day 7 were >71% mononuclear cells of various sizes. The rest of the cells could not be identified with certainty. It seemed therefore that cell recovery, numbers, type, and viability were comparable in BM and PB and would indicate that the results observed in Table 3 were not due to selective elimination or enrichment of a specific cell type.

Results of studies of T cells are shown in Table 4. Testing for suppressor T cell function in both BM and PB was accomplished by adding an enriched population of T cells from either compartment to autologous peripheral blood B cells. The latter were then tested for Ig synthesis after 7 days' culture in the presence of PWM. We have not tested the suppressor capacity of T cells on BM-B cells due to the poor Ig synthetic capacity of the latter (4% ± 2%). BM-T cells were more effective than PB-T cells (p < 0.05) in suppressing Ig synthesis.
Table 4. Suppressor T Cell Subpopulations in Human Bone Marrow and Peripheral Blood

<table>
<thead>
<tr>
<th>Cell Source (no. exp.)</th>
<th>Cells With Cytoplasmic Fluorescence* of Cell Cultures (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T</td>
</tr>
<tr>
<td>BM (6)</td>
<td>0</td>
</tr>
<tr>
<td>PB (8)</td>
<td>1 ± 1</td>
</tr>
</tbody>
</table>

*Different cell types cultured for 7 days with PWM and then stained by FITC antihuman Ig antiserum.
†B cells of PB were always used when cocultured with T cells.
‡p < 0.05 when compared to value when PB-T cells were used.

DISCUSSION

Our previous studies on human bone marrow enriched lymphocyte subpopulations showed that the BM compartment is different from the PB compartment in normal states,1-3 immune deficiency diseases,14 and certain hematologic malignancies.15 In this study, we have characterized the various cell subpopulations and the differentiation potentials of human BM lymphocytes from donors without demonstrable lymphocyte or immunologic abnormalities. Since BM cells are usually contaminated with some peripheral blood cells, we have attempted to minimize possible artifacts by collecting small BM samples of 1-2 ml and using the sucrose density gradient centrifugation technique2 to obtain the maximal yield of BM mononuclear cells. Moreover, it is difficult to obtain pure lymphocytes and separate them from other nonlymphocytic small mononuclear cells. At present there is no technique that gives pure BM lymphocytes.

In this study, identification of the various markers on BM lymphocytes showed that the majority (60% ± 13%) of human BM cells carried no known cell markers, and we chose to call them null cells. The B lymphocytes carrying surface membrane Ig constituted 34% of bone marrow lymphocytes and the majority carried IgM (Table 1). This observation supported our earlier findings in which we used an 125I-anti-Ig technique and those recently reported in the mouse.16 Not all marrow B lymphocytes carried receptors for C3 or Fc, indicating that bone marrow B cells may not be able to engage in the effector phase of the immune response which requires binding to immune complexes or complement. Marrow B cells are capable of binding to antigens1,2 and
therefore may be involved in the afferent (recognition) phase of the immune response. However, this capability was shown to be of extremely restricted potential\textsuperscript{17,18} and required a maturation phase in peripheral lymphoid organs.\textsuperscript{19} In comparing peripheral blood B cells to those of the BM, the former constituted 23\textsubscript{o} of lymphocytes and carried the three markers: Ig, C\textsubscript{3} receptors, and F\textsubscript{c} receptors. They therefore represented a fully mature differentiated B cell population.\textsuperscript{20-22} The numbers carrying IgG were greater than those carrying IgM or IgA. Since we have not used F(ab)\textsubscript{2} fragments of the anti-Ig we cannot exclude the possibility that the antiserum bound to the F\textsubscript{c} receptor and not to the Ig on blood lymphocytes.

T cells in the BM were about 6\textsubscript{o} of the BM lymphocytes. These BM-T cells have been shown to be part of the circulating pool\textsuperscript{23} and probably represented a specific subpopulation with preferential homing capacity to the BM.\textsuperscript{24} In comparing equal numbers of T cells for their suppressor activity, we have demonstrated that BM-T cells were more effective in suppressing B cell Ig synthesis than PB-T cells. This finding could be important in the control of B cell maturation and differentiation and in regulating B cell autoreactive clones within the bone marrow at an early stage of their differentiation. Preliminary studies of BM from two patients with systemic lupus erythematosus have demonstrated the absence of suppressor T cells in the bone marrow (unpublished observations).

Part of the bone marrow null cells may be B and T cell precursors and may represent a stage in the differentiation sequence between stem cells and mature B or T cells. In this study, we have shown that bone marrow cells cultured in vitro in the presence of pokeweed mitogen showed evidence of partial maturation by acquiring surface membrane Ig. The same cells were shown to be incapable of Ig synthesis (Table 3) and, therefore, they could not be considered as fully mature B cells.\textsuperscript{25} The observed increase of surface membrane Ig carrying cells in BM was probably not due to passive adsorption of Ig onto cells, since all cultures were in gamma globulin-free medium, and very few BM cells were shown to synthesize or secrete Ig (Table 3). Peripheral blood B cells when cultured in vitro can undergo terminal maturation by acquiring the capacity to synthesize Ig. This finding would indicate that PB-B cells are capable of differentiating into fully mature B cells that can be involved in the effector phase of the immune response by secreting Ig or antibody.\textsuperscript{1,20,25} The observed differences between BM and PB-B cell subpopulations in the 7-day culture were not due to selective elimination of nonlymphoid populations because cell recovery and viability were almost comparable in both cultures.

Thymosin has recently been shown to be capable of inducing T cell maturation of human BM cells.\textsuperscript{26} This observation would indicate that bone marrow T cell precursors require a thymic factor for their full maturation into T cells.\textsuperscript{22,26}

Our findings and those of several recent studies\textsuperscript{24,26,27} have clarified certain aspects of BM immune competence in the human and have emphasized the unique nature of the various lymphoid cells within the BM compartment. There are still several questions to be answered. Clarification of the maturation sequence of stem cells to null cells and probably to precursors of B and T cells is needed. Knowledge of the factors regulating B cell differentiation and emi-
BONE MARROW LYMPHOCYTES

429

gregation from the BM would clarify the mechanisms involved in BM-B cell homeostasis and autotolerance. It would be of interest to apply the information presented here to studies of bone marrow lymphocyte populations in the various immunologic and/or hematologic diseases suspected of having abnormalities of B cell differentiation or maturation.28

ACKNOWLEDGMENT

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REFERENCES

20. Osmond DG, Nossal GJV: Differentiation of lymphocytes in mouse bone marrow. I. Quantitative radioautographic studies of antiglobulin binding by lymphocytes in bone...
Human bone marrow lymphocytes: B and T cell precursors and subpopulations

NL Abdou, JB Alavi and NI Abdou