B-Cell Differentiation Following Autologous, Conventional, or T-Cell Depleted Bone Marrow Transplantation: A Recapitulation of Normal B-Cell Ontogeny

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The circulating lymphocytes of 88 consecutive patients following autologous, conventional, or T-cell depleted bone marrow transplantation were serially analyzed for B-cell surface antigen expression and function. In the majority of patients, except for those who developed chronic graft-versus-host disease, the number of circulating CD20 B cells normalized by the fourth posttransplant month. The earliest detectable B cells normally expressed CD19, surface immunoglobulin (slg), CD21, Leu-6, and lacked expression of CD10 (CALLA). In addition, the circulating B cells expressed CD1c, CD38, CD5, and CD23 for the first year following transplant, antigens that are normally expressed on a small percentage of circulating B cells in normal adults, but highly expressed on cord blood B cells. Similar to cord blood B cells, patient B cells isolated during the first year following transplant, proliferated normally to Staphylococcus aureus Cowan strain I (SAC), and produced IgM, but minimal or no IgG when stimulated with pokeweed mitogen and SAC, unlike normal adult B cells that produce both. The similar phenotype and function of posttransplant and cord blood B cells, and their similar rate of decline in patients and normal children adds further evidence to support the hypothesis that B-cell differentiation posttransplant is recapitulating normal B-cell ontogeny.

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nine healthy neonates, and peripheral blood from nine sibling donors (age 2 to 12 years, median 4) of patients undergoing therapeutic bone-marrow transplantation, and 85 healthy hospital or laboratory personnel. These studies were performed after informed consent was obtained under protocols approved by the Human Subjects Review Committee of Memorial Hospital and the Committee on Human Rights in Research of the New York Hospital-Cornell Medical Center.

**Cell preparations.** Heparinized blood was collected from patients every three to six months following bone marrow transplantation. Peripheral blood mononuclear cells (PBMC) were collected after centrifugation on Ficoll-Hypaque density gradients. Monocytes were removed from samples by plastic adherence at 37°C for 1 hour.

**Immunofluorescence analysis.** Directly fluoresceinated (FITC) HLE-1 (CD45, common leukocyte antigen), Ms IgG, Leu-16 (CD20, pan-B cell), CALLA (CD10, pre-B cell), and phycoerythrin-conjugated (PE) Ms IgG, Leu-1 (CD5), Leu-4 (CD3, pan-T cell), Leu-16, Leu-17 (CD38), HLA-DR, Leu-8, Leu-19 (CD56, natural killer (NK) cell) and unconjugated Leu-5 (CD2, sheep red blood cell receptor, pan-T cell and NK cell) were purchased from Becton Dickinson (Mountain View, CA). FITC-conjugated B4 (CD19, pan-B cell), B2 (CD21, B-cell subset marker), Mo2 (monocyte-specific) and PE-conjugated TQ1 were obtained from Coulter Immunology (Hialeah, FL). FITC-conjugated antibody reactive with sIg and unconjugated OKT10 (CD38) were purchased from Ortho Diagnostic Systems (Raritan, NJ). M241 (CD1c) and PL13 (CD23), were generously supplied by Dr Robert Knowles. Two-color direct immunofluorescence was performed on monocyte-depleted peripheral blood lymphocytes using standard techniques. Two-color immunofluorescence utilizing direct PE-conjugated and indirect FITC-conjugated reagents required three separate antibody incubations, with two washes between incubations. Cells were treated sequentially with the mouse anti-human unconjugated antibody, followed by the FITC-conjugated goat anti-mouse reagent. The final incubation was with the directly conjugated phycoerythrin mouse anti-human monoclonal antibody. This order was utilized to prevent non-specific binding of the FITC-conjugated goat anti-mouse antibody to the PE-conjugated mouse anti-human monoclonal antibody.

**Immunofluorescence was performed on isolated monocyte-depleted peripheral blood lymphocytes or on whole blood.** The whole-blood technique consisted of the addition of appropriate amounts of undiluted conjugated antibody to 50 μl of whole blood, followed by a half-hour incubation at room temperature. Red cells were then lysed with a 1/10 dilution of FACS lysis solution (Becton Dickinson), followed by two washes. Immunofluorescence samples were analyzed on an EPICS C-cell sorter (Coulter Immunology) or a FACScan (Becton Dickinson).

**In vitro B-cell mitogen assays.** 1 x 10⁵ monocyte-depleted peripheral blood lymphocytes were stimulated with 0.01% *Staphylococcus aureus* Cowan strain I (SAC) (Pansorbin, Calbiochem, La Jolla, CA) or rabbit anti-human IgM (anti-μ) coupled to polyacrylamide beads (4, 2, or 1 μg/ml) in RPMI-1640 supplemented with 25 mmol/L Hepes buffer, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10⁻³ M 2-mercaptoethanol, and 10% fetal bovine serum (Hazelton, Lenexa, KS). Cultures were maintained for 72 hours at 37°C in 5% humidified CO₂. Cells were labeled with 1 μCi/well of tritiated thymidine (6.8 μCi/mmole/L) (New England Nuclear, Boston, MA) during the last 24 hours of culture. The results are expressed as the mean counts per minute (cpm) of triplicate cultures minus the unstimulated medium control.

**Immunoglobulin production assay.** 2 x 10⁵ PBMCs per well were co-cultured with a synergistic combination of PWM (1:4000) and SAC (0.004%) for 7 days as previously described. Immunoglobulin secreted into the supernatant of replicate wells was quantitated in duplicate by an inhibition enzyme-linked immunosorbent assay (ELISA), based on the method of Voller et al. The amount of Ig in the test sample was calculated from the regression line of the Ig
standard. The results are expressed as nanograms per milliliter of immunoglobulin produced. The sensitivity of the assay is approximately 10 ng/mL of Ig.

Statistical analysis. Serial observations for cell surface markers and in vitro functional responses were recorded for each patient at 3 to 6 month intervals. To determine if results between transplant groups were the same, a non-parametric Kruskal Wallis test was performed within each of the time intervals. As a result of using multiple significance tests, the Bonferroni inequality was implemented. If the test in any time interval produced a P-value less than .01, then the hypothesis of no difference between the transplant groups was rejected for that time interval.

RESULTS

Serial phenotypic analyses performed on the circulating lymphocytes of 88 patients following T-cell depleted (Lectin group), conventional, or autologous bone marrow transplantation revealed that the number of CD20+ B cells rapidly increased after the third month following each of the three types of bone marrow transplantation (Fig 1). At time points greater than three months, most patients, except those with chronic GVHD, had normal or greater than normal numbers of circulating B cells. Patients with cGVHD accounted for

85% of the recipients of conventional transplants who had less-than-normal numbers of circulating B cells after the third transplant month. Patients with cGVHD had significantly fewer numbers of CD20+ cells from 3 to 9 months post transplant (P < .0002 at 3 to 6 months, P < .015, 6 to 9 months). By 9 months post BMT, these differences were no longer significant (P > .14).
The circulating CD20+ cells normally expressed HLA-DR, sig, CD19 (B4), CD21 (B2), TQ1, Leu-8, and lacked expression of CD10 (CALLA) at all time points post transplant. This is demonstrated in Fig 2, in a representative analysis of the CD20+ cells of a patient 3.5 months following a conventional bone-marrow transplant. Except for rare patients (<5%), no differences could be demonstrated between the expression of these eight antigens on the earliest detectable B cells and those cells analyzed at later time points posttransplant (data not shown). In contrast to the results observed with these B-cell markers, the percentage (and absolute number) of CD5+ B cells was above normal levels in the majority of patients during the first nine months post transplant (Fig 3), as shown in the two-color histograms performed on a patient 6 months following a conventional transplant (Fig 4). These profiles demonstrate that CD5 antigen density on the surface of B cells is low in contrast with the high-antigen density of CD5 on the CD3+ T cells. After the first nine months posttransplant, there was a gradual decline to the more normal levels that were detected by the second posttransplant year (Fig 3). There were no significant differences among the three transplant groups in this pattern of CD5 B-cell expression (P > .01).

The expression of CD1c (M241), CD38 (OKT10), and CD23 (PL13) on circulating B cells posttransplant is shown in Fig 5. Whereas the majority of circulating B cells during the first 6 to 12 months post BMT are CD1c+, CD38+, and CD23+, the expression of these antigens markedly decreases during the second year posttransplant. No appreciable differences in the expression of these antigens among the three transplant groups were appreciated. As shown in Fig 5, these antigens are also strongly expressed on the circulating B cells of healthy neonates, but expression decreases with age, such that the B cells of older children (>2 years) and healthy adults are predominantly CD1c-, CD38-, and CD23- negative. The decline of CD1c, CD38, and CD23 expression, normally observed in the posttransplant period, is illustrated in the histograms in Fig 6, which represent analyses performed on the circulating lymphocytes of a patient 4 and 18 months after a T-cell depleted transplant, as well as parallel studies of B cells of a healthy neonate and adult (Fig 6B).

In vitro responses to the T-cell independent mitogen SAC demonstrated that co-incident with the return of circulating B cells 3 to 6 months posttransplant, the SAC response normalized in the majority of patients (Fig 7). There were no significant differences among the transplant groups (P > .01). Five of 8 patients who had an abnormal SAC response more than three months after a conventional transplant had cGVHD, and generally had lower-than-normal numbers of circulating B cells. Once the SAC response normalized, the anti-μ response was also within the normal range in 73%, 86%, and 93% of samples following an autologous, conventional, or T-cell depleted transplant, respectively.

Analysis of in vitro immunoglobulin production, utilizing a synergistic combination of PWM and SAC, demonstrated

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**Fig 4.** Two-color direct immunofluorescence profile performed on the circulating lymphocytes of a patient 6 months following a conventional BMT. Left: Mutually exclusive double-positive control: CD3-FITC (pan-T cell)/CD56-PE (NK cell marker). Middle: CD3-FITC/CD5-PE. Right: CD20-FITC/CD6-PE.
Fig 6. (A) Two-color immunofluorescence profiles performed on the circulating B lymphocytes of a patient 4 and 18 months following a T-cell depleted BMT. Left, CD1c-FITC/CD20-PE; Middle, CD38-FITC/CD20-PE; Right, CD23-FITC/CD20-PE. (B) Two-color immunofluorescence profiles demonstrating CD1c, CD38, and CD23 expression on the B cells of cord blood (upper panels) and a healthy adult (lower panels).
Several studies have demonstrated intrinsic B-cell dysfunction following bone-marrow transplantation, as evidenced by sub-optimal responses of isolated B-lymphocytes to B-cell mitogens and growth factors, as well as the failure of purified B cells to terminally differentiate, despite the addition of normal T cells or T-cell–derived growth factors. During the first transplant year, it is likely that increased T-cell suppression and lack of appropriate T-cell help contribute to the observed deficiencies of B-cell function. However, the contribution of these factors during the second year posttransplant is less clear. By this time, in most patients lacking cGVHD, the CD4/CD8 ratio, and absolute numbers of helper inducer cells (CD4+ CD29+) and suppressor cells (CD8+ CD57+ CD11b+) have begun to normalize. Small et al, manuscript in preparation). In addition, in vitro studies have demonstrated the ability of these T cells to adequately provide B-cell help. This present study demonstrates that the earliest B cells reconstituting the peripheral blood post BMT share unique phenotypic and functional characteristics with the B cells of cord blood, regardless of the type of transplant given.

The earliest detectable circulating B cells expressed HLA-DR, sig, CD19, CD20, CD21, Leu-8, and TQ1, as do normal adult peripheral B cells. In addition, for the first nine months posttransplant, our patients demonstrated an absolute increase in CD5+ B cells that gradually decreased in the second posttransplant year. Although CD5+ B cells are a minor population of the sIg+B cells in normal adults, increases in CD5+ B cells have been seen in fetal lymphoid organs, cord blood, and young infants. Our findings post BMT are similar to those of Ault et al and Antin et al but contrasts to other studies in which CD5+ B cells were not detected in individuals receiving conventional or monoclonal antibody T-cell depleted bone marrow. Differences in marrow purging, preparative regimen, or immunofluorescence technique may affect the numbers of CD5+ B cells detected. Nevertheless, an increase in CD5 positive B cells during the first year posttransplant with subsequent decline in the second year after transplant was consistently observed in our patients. These results were based on studies of individual patients serially tested on multiple occasions and were seen whether the staining was performed on whole blood or on purified monocyte-depleted lymphocytes.

Recently, it was reported that B cells isolated from individuals studied during the first 7 months posttransplant (n = 7) expressed increased levels of CD23 in association with decreased Leu-8 and CD19 expression, unlike normal controls and patients tested greater than ten months posttransplant. Although normal B cells demonstrate an increase in CD23 expression and loss of Leu-8 expression with activation the early B cells of these patients showed abnormalities of B-cell activation, proliferation, and differentiation, even in the presence of low molecular weight B-cell growth factor, the proposed ligand of CD23.

Discussion

Several studies have demonstrated intrinsic B-cell dysfunction following bone-marrow transplantation, as evidenced by sub-optimal responses of isolated B-lymphocytes to B-cell mitogens and growth factors, as well as the failure of purified B cells to terminally differentiate, despite the addition of normal T cells or T-cell–derived growth factors. During the first transplant year, it is likely that increased T-cell suppression and lack of appropriate T-cell help contribute to the observed deficiencies of B-cell function. However, the contribution of these factors during the second year posttransplant is less clear. By this time, in most patients lacking cGVHD, the CD4/CD8 ratio, and absolute numbers of helper inducer cells (CD4+ CD29+) and suppressor cells (CD8+ CD57+ CD11b+) have begun to normalize, Small et al, manuscript in preparation). In addition, in vitro studies have demonstrated the ability of these T cells to adequately provide B-cell help. This present study demonstrates that the earliest B cells reconstituting the peripheral blood post BMT share unique phenotypic and functional characteristics with the B cells of cord blood, regardless of the type of transplant given.

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patients demonstrated an increase in CD23 expression during the first year posttransplant, serial comparison of Leu-8 and CD19 expression on the B cells of individual patients analyzed at early and late time points, revealed no differences from normal controls. The detection of CD23 on normal resting cord blood B cells suggests that the expression of this antigen may not always reflect B-cell activation. In this respect, CD23 resembles the discontinuous expression of CD38 during normal B-cell ontogeny, which is seen not only on activated B cells, but also on resting fetal B cells, circulating cord blood B cells, as well as terminally differentiated plasma cells, with minimal expression on resting, circulating adult B cells.\textsuperscript{14,48,49}

The CD1 antigens were originally detected on normal cortical thymocytes, dermal dendritic cells, and acute T-cell leukemia cell lines.\textsuperscript{50-52} Of the CD1 antigen cluster, only CD1c (M24) but not CD1a (T6) or CD1b (4A76) has been demonstrated serologically and biochemically on B cells.\textsuperscript{53} CD1c additionally differs from CD1a and CD1b by the size of its heavy chain, association with a 16-Kd polypeptide, presence on dermal dendritic cells, and lack of association with CD8 on thymocytes.\textsuperscript{28,54-56} The distribution of CD1c positive B cells in normal adults has included approximately half of splenic B lymphocytes, follicular mantle zone B cells, and a small fraction of circulating B cells.\textsuperscript{14,43,57} Normal cord blood B cells are predominantly CD1c positive, with subsequent decline of CD1c antigen expression on peripheral blood B cells by two to three years of age.\textsuperscript{14,44} This rate of decline in young children parallels that seen after transplantation. Although the role of CD1c during normal B-cell ontogeny is currently unknown, recent data have demonstrated that CD1c may act as a target molecule for T cells bearing the gamma-delta T-cell receptor.\textsuperscript{58}

Our studies of the functional capabilities of patient B cells during the first year posttransplant demonstrate normal responsiveness to SAC, normal IgM production, and markedly impaired IgG secretion in most patients, similar to that seen when cord blood B cells are analyzed. Apart from patients with cGVHD, no significant differences were seen among the three transplant groups. Similar to other studies performed on patients receiving conventional or chemotherapy-purged autologous marrow, poor B-cell function was consistently demonstrated during the first three months posttransplant, when the numbers of circulating B cells are low. In general, most in vitro B-cell functions normalize by 1 to 3 years posttransplant, except in those patients who develop cGVHD.\textsuperscript{2,5,7,8,21} The early normalization of our patients SAC and anti-\(\mu\) response, and in vitro IgM production differs from some studies in which abnormal proliferation and differentiation was demonstrated when T-cell de-
pleted, B-cell enriched populations were analyzed. In these reports although T cells were removed, significant percentages of monocytes remained (50% to 70%), which may suppress in vitro patient B-cell responses and account for these observed differences.

Although the ability to secrete IgM in response to a variety of polyclonal B-cell activators is present at birth, the ability to produce IgG is gradually attained between birth and two years of age. In our transplant patients, the in vitro ability to secrete IgG in response to PWM and SAC is acquired by most patients between 12 and 24 months posttransplant. Although serum IgG1, IgG3, and IgM levels have been shown to normalize in most patients without cGVHD during the first year posttransplant, deficiencies of IgA, IgG2, and IgG4 may persist late after transplant. This pattern of acquisition of serum immunoglobulins is similar to that seen in normal children. This in vivo data is consistent with in vitro studies performed on B cells from patients 3 to 14 months following T-cell depleted BMT, in which a paucity of IgG2 and IgG4 positive plasma cells were produced after stimulation with PWM or lipopolysaccharide. The failure of normal T cells to correct this defect may reflect the failure of posttransplant B cells to respond to T-cell factors involved in isotype-switching.

In this report we have demonstrated that B cells in the early posttransplant period share a number of phenotypic and functional similarities with B cells found in cord blood. Integrating our present results with previous observations that normal T cells cannot correct the defective function of engrafted B cells, lends further strength to the concept that B cells posttransplant are initially intrinsically limited in their functional capabilities. In normal individuals, as in transplant patients, CD1c, CD5, CD38, CD23 positive B cells become a minor subset as a person develops humoral immune competence.

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REFERENCES

reconstitution following bone marrow transplantation: Comparison of recipients of T-cell depleted marrow with recipients of conventional marrow grafts. Blood 73:1340, 1989


35. Atkinson K: T cell subpopulations defined by monoclonal antibodies after HLA-identical sibling marrow transplantation. II. Activated and functional subsets of helper-inducer and cytotoxic-suppressor subpopulations defined by two-colour fluorescence flow cytometry. Transplantation 1:121, 1986


52. Van de Rijn M, Lerch PG, Bronstein BR, Knowles RW, Bhan AK, Terhorst C: Human cutaneous dendritic cells express two glycoproteins T6 and M241 which are biochemically identical to those found on cortical thymocytes. Hum Immunol 9:201, 1984


54. Knowles RW: Immunohistochemical analysis of the T-cell specific antigens, in Reinherz EL, Haynes BF, Nadler LM, Bernstein ID
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