Deficient Expression of Adhesion Molecules by Human CD5⁻ B Lymphocytes Both After Bone Marrow Transplantation and During Normal Ontogeny

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Despite the relatively early reconstitution of blood B-lymphocyte counts observed in patients treated with bone marrow transplantation (BMT), these patients undergo a prolonged phase of humoral immunodeficiency. Adhesion molecules perform relevant functions in many cell types. The present study examines the expression of several adhesion molecules on human B lymphocytes newly formed after BMT. Blood B cells from 38 patients were studied by flow cytometry and three-color analysis. Blood CD5⁻ B lymphocytes obtained at an early stage after BMT (2 to 4 months) showed a markedly low expression of the adhesion molecules CD54, CD44, CD11a, and CD62L. However, these cells exhibited a normal expression of other molecules including CD29, CD19, CD20, and DR. This deficiency was progressively corrected, reaching normal levels in the late post-BMT period (12 to 15 months). In contrast, CD54, CD44, CD11a, and CD62L expression on the patients' CD5⁻ B lymphocytes was found to be consistently normal. Deficient adhesion molecule expression on CD5⁻ B cells in the early post-BMT period was similarly observed in patients treated with either an allo-BMT (n = 24) or an auto-BMT (n = 14). Because the post-BMT period mimics normal ontogeny, adhesion molecule expression was also investigated in cord-blood B lymphocytes. Cord-blood CD5⁻ B lymphocytes, in contrast to CD5⁺, also expressed CD54, CD44, CD11a, and CD62L at levels much lower than those found in normal adults. Present data suggest that progressive expression of CD54, CD44, CD11a, and CD62L seems to be a part of the maturational program of CD5⁻ B lymphocytes during both post-BMT and normal development periods. This observation may help to explain the humoral immunodeficiency observed in both conditions.

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MATERIALS AND METHODS

Patients. Thirty-eight durably engrafted patients who had received therapeutic allogeneic or autologous BMT were included in this study. Twenty-four were male and 14 were female. Patients ages ranged from 5 to 38 years (median, 22.6 years). Fourteen patients received an autologous transplant for either acute myelogenous leukemia (AML) (n = 5), acute lymphoblastic leukemia (ALL) (n = 5), or non-Hodgkin's lymphoma (NHL) (n = 4). Twenty-four patients received a HLA-identical allogeneic transplant for either AML (n = 4), ALL (n = 10), aplastic anemia (AA) (n = 4), chronic myelogenous leukemia (CML) (n = 3), NHL (n = 1), thalassemia major (n = 1), or Fanconi anemia (n = 1). Pretransplant conditioning was as follows: patients with ALL, AML, and CML were conditioned with either busulfan and cyclophosphamide (CY) (n = 11).

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Fig 1. Study of adhesion molecule (CD11a in this case) expression on CD5- and CD5+ B lymphocytes by three-color analysis and flow cytometry. Mononuclear cells were purified from the blood of a patient, obtained 4 months after BMT, and the cells were stained for the indicated antigens. (A) Forward-scatter versus side-scatter dot-plot analysis. (B) An R1 region selecting CD19+ lymphocytes was drawn in a side-scatter versus FL3 (CD19-PercP labeling) dot plot. (C) Cells contained in R1 (CD19+) were classified as CD5+ (R2) and CD5- (R3) in an FL2 (CD5-PE labeling) versus FL3 (CD19-PercP) dot plot. (D) Histogram of FL1 (CD11a-FITC) expression on R3-gated (CD19+CD5-) cells. (E) Histogram of FL1 (CD11a-FITC) expression on R2-gated (CD19+CD5+) cells.

or fractionated total body irradiation (TBI) and CY (n = 16); NHL cases received carmustine, CY, and VP-16; the case of thalassemia was conditioned with busulfan and CY; AA patients received CY, procarbazine, and antithymocyte Ig; the case with Fanconi anemia was conditioned with CY alone. Prophylaxis of graft-versus-host-disease (GVHD) in patients treated with allo-BMT was performed with methotrexate and cyclosporine. None of these patients developed either acute GVHD greater than grade I or chronic GVHD. Blood samples were taken from all the patients at early (2 to 4 months) and late (12 to 15 months) post-BMT periods. Additional blood samples were obtained bimonthly from 6 patients over the subsequent two years. Healthy volunteers were used as controls. Cord-blood samples were obtained from 16 clinically healthy neo-

Fig 2. Expression of CD54, CD44, CD11a, and CD62L on CD5- (A) and CD5+ (B) B lymphocytes obtained from healthy controls (○) and BMT-treated patients studied at early (2 to 4 months, □) and at late (12 to 15 months, △) periods after BMT. Values shown are the percentages of B cells positive for the indicated molecule, and the results represent the mean ± SD of 38 BMT-treated patients and 23 healthy volunteers.
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Fig 3. An example of histogram representation of CD54, CD44, CD11a, and CD62L expression on CD5– (A) and CD5+ (B) B lymphocytes obtained from a BMT-treated patient, 3 months (early post-BMT period) and 14 months (late post-BMT period) after BMT.

nates. All patients and controls were informed about the objectives and methods of the study and gave their consent.

Monoclonal antibodies (MoAbs). The following fluorochrome (fluorescein isothiocyanate, FITC; phycoerythrin, PE; and peridinin chlorophyll protein, PerCP)-labeled mouse MoAbs were used in this study: control IgG1-FITC, IgG1-PE, IgG2-FITC, IgG2-PE and IgG1-PerCP, and anti-CD11a-FITC, anti-CD11b-PE, anti-CD11c-PE, anti-CD19-PerCP, anti-CD20-FITC, anti-CD20-PerCP, anti-CD25-PE, anti-CD44-FITC, anti-CD54-PE, anti-CD62L-FITC, anti-CD62L-PE, anti-CD69-FITC, and anti-CD71-FITC MoAbs were purchased from Becton Dickinson (San Jose, CA); anti-CD5-FITC, anti-CD5-PE, anti-CD19-FITC, anti-CD19-PE, anti-CD29-PE, and anti-DR-FITC MoAbs were obtained from Coulter (Hialeah, FL).

Cell preparation. Venous or cord-blood samples were drawn into heparinized vacutainer tubes, and the mononuclear cells were isolated on a Ficoll density gradient (ICN Biomedicals Inc, Costa Mesa, CA). Cells obtained from the interface were washed three times in Hanks’ balanced salt solution (HBSS) and resuspended at 5 × 10⁶ cells/mL. Viability of the isolated cells always exceeded 95% as determined by trypan blue exclusion.

Staining of cells. All antibodies were spun at 12,000g for 15 minutes at 4°C to remove antibody aggregates that could cause nonspecific staining of lymphocytes. In single-, double-, and triple-labeling experiments, 5 × 10⁵ blood mononuclear cells were incubated with 10 μL of FITC-, PE-and/or PerCP-conjugated MoAb for 30 minutes at 4°C. The cells were washed twice in HBSS containing 0.1% sodium azide and analyzed within 2 hours.

Flow cytometry. Flow cytometry analysis was performed on a standard FACScan instrument (Becton Dickinson) equipped with an air-cooled argon ion laser, which operated at 488 nm and 15 mW of power. The instrument was equipped with three fluorescence-detector
The percentages of CD5- B cells positive for the indicated molecule, CD1, CD44, CD11a, and CD62L on CD5- B lymphocytes obtained from allo-BMT-treated and auto-BMT-treated patients, at early period (2 to 4 months, A) and at late (12 to 15 months, B) periods post-BMT. Values shown are the percentages of CD5- B cells positive for the indicated molecule, and the results represent the mean ± SD of 24 and 14 patients treated with allo- and auto-BMT, respectively.

Statistical analysis. The Mann-Whitney rank-sum test was used to compare adhesion molecule expression results. Differences with a P value < .05 were considered significant.

RESULTS

Adhesion molecule expression on blood CD5- and CD5+ B-lymphocyte subsets reconstituted after BMT. Blood B lymphocytes from BMT-treated patients were obtained at early (2 to 4 months) and late (12 to 15 months) time intervals, after the transplant, and the expression of several surface molecules was analyzed on CD5+ and CD5- B-cell subsets. The study was performed on isolated mononuclear cells that were stained with CD19-PerCP MoAb and FITC- or PE-conjugated anti-CD5 MoAb and PE- or FITC-conjugated MoAb directed against several adhesion molecules, and the analysis was performed as follows: (1) cells were gated for CD19+ lymphocytes using side scatter versus log fluorescence plot (Fig 1A and B); (2) CD19+ B lymphocytes were gated into two nonoverlapping subsets according to their CD5 expression: CD19+CD5+ and CD19+CD5- cells (Fig 1C, R2 and R3, respectively); (3) adhesion molecule expression was analyzed for cells within either the CD19+CD5+ (R2) or the CD19+CD5- (R3) gate (Fig 1D and E). A minimum of 5,000 B-cell–gated events were collected for analysis of adhesion molecule staining. Control-stained cells were also gated according to CD19 CD5 expression and, thus, differed for each subset. The cursor was set so that less than 1% of the cells stained positively with the corresponding control antibodies. The percentage of cells that stained positively and the mean fluorescence intensity were recorded for each sample.

As can be seen in Figs 2A and 3A, CD5- B lymphocytes obtained in the early post-BMT phase exhibited a considerably low expression of the adhesion molecules CD54, CD44, CD11a, and CD62L in comparison with that observed in the same population of healthy controls (P < .0001, < .0001, < .0001, and < .0005, respectively). The expression on this B-cell subset of other adhesion molecules, such as CD29, as well as the expression of other B-cell antigens, including CD20 and DR, did not show this alteration (data not shown). Similar results were obtained when the detection of CD20, rather than CD19, antigen was used to identify B lymphocytes. The expression of all four adhesion molecules under study returned to normal in the late post-BMT period (Figs 2A and 3A). None of these B-cell populations showed substantial numbers of activated lymphocytes, defined as CD25+, CD69+, and CD71+ cells.

It has been reported previously that blood samples obtained during the early post-BMT interval show a large proportion of B cells expressing the CD5 molecule. In our study, the proportion of CD5- B cells in early post-BMT blood samples accounted for 42% ± 6% of the total B lymphocytes whereas, in healthy controls, only 18% ± 3% of the B cells expressed CD5 (mean ± SD of 38 and 23 different cases, respectively). The CD5- B-cell percentage decreased in the late post-BMT period (to 31% ± 5%, mean ± SD, n = 38), but still remained higher than normal. Figures 2B and 3B summarize the analysis of adhesion molecule expression on these B lymphocytes. In contrast to CD5- B cells, the expression of CD54, CD44, CD11a, and CD62L on CD5+ B lymphocytes was found to be normal even in the early post-BMT period.

Figure 4 shows the comparison of adhesion molecule expression by CD5- B cells between auto- and allo-BMT–treated patients. As can be seen, no difference was observed between the two groups of patients when the data from either early (Fig 4A) or late (Fig 4B) post-BMT periods were compared.

To examine in detail the sequence of normalization of altered adhesion molecule expression in the post-BMT period, CD5- B cells from several patients were analyzed bimonthly (Fig 5). Follow-up studies showed that the initial low expression of CD54, CD44, CD11a, and CD62L was progressively corrected during the following months. The time taken for complete recovery of adhesion molecule expression varied from patient to patient, ranging between 6 and 14 months in the 6 cases examined (10.6 ± 3 months; mean ± SD).

Comparative analysis of adhesion molecule expression by B lymphocytes from cord and adult blood. Because B-cell reconstitution after BMT probably mimics physiological B-
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Cell ontogeny, the possibility that the described alterations also occurred in the cord-blood B lymphocytes was examined. Figure 6 shows that cord-blood CD5- B cells also exhibited an expression of CD54, CD11a, and CD62L much lower than that observed in adult CD5- B lymphocytes (P < .0001, <.0003, and .0001, respectively). In addition, most cord-blood CD5- B lymphocytes expressed CD44, but at an intensity notably lower than that of adult CD5- B cells (P < .0001) (Fig 6B). On the other hand, cord-blood CD5+ B lymphocytes showed a normal expression level of all the adhesion molecules under study (data not shown).

DISCUSSION

Surface antigen expression profiles of blood B lymphocytes regenerating after BMT have been previously analyzed. However, these reports did not pay much attention on the adhesion molecule expression by the two major subsets of B lymphocytes. The present study shows that, early after BMT, newly formed CD5- B lymphocytes exhibit a markedly low expression of several adhesion molecules, such as CD54, CD44, CD11a, and CD62L, which are usually present on most adult normal CD5- B lymphocytes. This phenomenon seems to be restricted to certain molecules, because the presence of CD29, DR, CD19, and CD20 on the same cells remained unaffected. Sequential analysis of blood CD5- B lymphocytes after BMT shows that the low expression of the adhesion molecules under study gradually recovered, until normal levels were reached in the late post-BMT period (12 to 15 months post-BMT). The deficit was detectable for a variable, but usually prolonged, period of time. Moreover, this sequence of events was similarly observed after both auto- and allo-BMTs, thus ruling out a possible implication of allogeneic reaction in this phenomenon. Therefore, the low expression of CD54, CD44, CD11a, and CD62L on CD5- B lymphocytes, as well as its later correction, appear to be a general rule in the post-BMT regeneration of these B lymphocytes.

It has been proposed that post-BMT B-cell formation resembles B-cell ontogenic development during fetal and newborn life. This is based on the fact that both conditions show

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**Fig 5.** Sequential normalization of CD54, CD44, CD11a, and CD62L expression on CD5- B lymphocytes after BMT. The three upper rows show histogram representation of these molecules in samples from the same BMT-treated patient obtained at 3, 9, and 15 months after BMT. Bottom row shows results obtained with a normal control. The percentage of positive cells is included in the right upper angle of each histogram.
relevant similarities, including the temporary increase of CD5^+ B cells, the prolonged period of in vivo and in vitro B-cell disfunction, and the increased susceptibility to bacterial infections. In consequence, we examined the adhesion molecule expression on cord-blood B cells. The results showed that cord-blood CD5^- B cells also exhibited a similar abnormally low expression of CD54, CD44, CD11a, and CD62L on their surface. These data support the notion that the acquisition of a normal expression of these molecules on CD5^- B cells might be developmentally programmed. This idea is reinforced by the observation that some of these molecules (CD44, CD11a, and CD62L) show similar kinetics of expression during the normal B-cell development within the BM.

As has been previously described and also shown here, increased numbers of CD5^+ B lymphocytes are detectable after BMT. The reason for this increase remains unknown. Functionally, this B-cell subset (also known as B1a lymphocytes) has been commonly associated with natural auto-reactivity, whereas most CD5^- B lymphocytes (the so-called conventional or B2 lymphocytes) appear related to classical humoral immune response to foreign antigens. In this context, the result that diminished adhesion molecule expression occurs in early post-BMT and in cord-blood CD5^- B lymphocytes, but not in CD5^+, probably indicates that the two B-cell subsets exhibit significant differences in their maturational programs.

Increasing evidence support the view that adhesion molecules such as LFA-1 (CD11a-C18), CD54, CD44, and
CD62L are necessary for B lymphocytes to perform a broad variety of functions. Thus, these molecules appear to be required for efficient B–T-cell cooperation in several in vitro and in vivo models of B- and T-cell activation, growth, and differentiation,36,37 for B-cell–endothelial cell contact leading to migration and homing to different locations,16 and for the interaction between B lymphocytes and follicular dendritic cells, which seem to participate in memory B-cell selection.36,37 Accordingly, decreased expression of these molecules might impair many aspects of B-cell physiology. In this regard, it has been previously reported that infants (<2 years of age)38 as well as BMT recipients (<300 days post-BMT),39 show an immature splenic marginal zone, a B-cell area apparently associated with memory B-cell accumulation and humoral response to encapsulated bacteria.40 It could reasonably be predicted that a lack or insufficiency of relevant adhesion molecules hampers CD5– B lymphocytes migration into specialized areas. Therefore, the finding that CD54, CD44, CD1a, and CD62L are poorly expressed by circulating CD5– B lymphocytes, in the post-BMT period as well as in newborns, might contribute to explain the prolonged state of humoral immunodeficiency observed in both situations.

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