Peripheral blood CD5-positive B lymphocytes (B-1a cells) after allogeneic stem cell transplantation for acute myeloid leukaemia in humans

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Background. Only few data are available in literature regarding the reconstitution of B-1a cells after allogeneic bone marrow transplantation performed for haematological malignancies.

Methods. In this study we used flow cytometry to assess the reconstitution of the peripheral blood B-1a cell compartment after allogeneic peripheral blood stem cell transplantation. Cytometric analyses were performed over time on 11 consecutive patients undergoing allogeneic peripheral blood stem cell transplantation for acute myeloid leukaemia in our Haematology Unit and the results were compared with available data regarding B-1a cell reconstitution after allogeneic bone marrow stem cell transplantation.

Results. In spite of an earlier recovery of B-1a cells in the peripheral blood after allogeneic bone marrow transplantation, the reconstitution of this B-cell subset was similar, regardless of the source of stem cells employed.

Conclusions. Further studies are necessary in order to clarify the origin of B-1a cells in humans in health and illness.

Keywords: B-1a cells, reconstitution, stem cell transplantation.

Introduction

The B-lymphocyte cell compartment is characterised by expression of CD19 surface antigen, which is present early on bone marrow progenitor cells and persists throughout all stages of B-cell maturation[1]. This population of cells comprises subgroups with both phenotypic and functional differences. In adult humans, the majority of peripheral blood B cells are of the B-2 cell type, or conventional B cells, which account for about 10% of peripheral blood lymphocytes in humans[2]. The remaining B cells are described as B-1 cells, which constitute the majority of B cells in foetal life and infancy[3], and can be distinguished by the presence of CD5 into B-1a (CD5 positive) and B-1b (CD5 negative) subgroups[4-6].

As regards the different functional activities of these B-cell populations, B-2 lymphocytes are largely mediators of adaptive immunity and are located in the follicular zone; they respond to conventional protein antigens, with the help of T cells, and undergo antibody class switching and affinity maturation[7]. B-1 cells are considered to be effectors of innate immunity; they respond to T-cell-independent antigens, mainly carbohydrates, common to various infectious agents. B-1a cells spontaneously secrete IgM (natural antibodies)[4], while the antibody production by B-1b cells is induced and long-lasting.[8]

B-2 cells develop from late neonatal life onwards, are negatively selected by recognition of self-antigens and are generated in bone marrow throughout life[9].

In mice B-1 progenitors are initially present in the liver and can later be found in the spleen and less frequently in bone marrow[10]. They are positively selected by recognition of self-antigens[11] and reside mainly in the peritoneal and pleural cavities; they persist principally, if not exclusively, by self-replenishment[12].

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B-1 and B-2 cells are believed to derive from different, committed precursors and to be the end-product of two lineages[13,14], although an alternative suggestion is that differences in the repertoire of B-1 and B-2 cells are the result of antigen-driven selection[15]. While the origin of B-2 and B-1 lymphocytes is still being debated, few data are available on the reconstitution of the peripheral blood B-1a cell subset after allogeneic transplantation.

In humans, information concerning peripheral blood B-1a cells after transplantation has mainly been derived from the evaluation of minimal residual disease in patients with B-cell chronic lymphocytic leukaemia (B-CLL)[16-18], a disease in which the cells typically express CD5. However, physiological B-1a cells are distinguishable from the abnormal CD19+CD5+ B-CLL cells by different fluorescence patterns ($CD19^{+bright}$, $CD5^{+dim\text{-}to\text{-}bright}$ and $CD19^{+dim}$, $CD5^{+dim}$, respectively). Very few data are available regarding the reconstitution of B-1a cells after allogeneic bone marrow transplantation (BMT) performed for haematological malignancies other than B-CLL[19].

The aim of the present study was to evaluate peripheral blood B-1a cells by cytometric analysis in humans after allogeneic peripheral blood stem cell transplantation (SCT) performed for haematological neoplasms other than B-CLL. The results were also compared with the published data on B-1a cell recovery after allogeneic BMT.

Patients and methods

1. *Patients.* As from January 2002 to May 2006, B-1a cell assessment by cytometric analysis was performed in 52 peripheral blood samples from 11 consecutive patients who underwent allogeneic SCT for acute myeloid leukaemia, other than leukaemia with inv(16)(p13q22) or t(15;17(q22;q12), in the Bone Marrow Transplant Unit of the Haematology Section of Verona University. Two of the 11 cases had leukaemia secondary to transformation of a myelodysplastic syndrome. Cytometric analyses were performed on days 30 (11 patients), 90 (11 patients), 180 (11 patients), 360 (9 patients), 550 (5 patients) and 700 (5 patients) after transplantation. All cases in which relapse occurred were excluded from evaluation.

   The median age of the patients was 42.6 years (range, 20-65 years). The study population consisted of nine males and two females (male:female ratio 4.5:1).
2. *Preparative regimens*. The allogeneic SCT was from related donors in eight cases and from unrelated donors in the remaining three cases. **Myeloablative** conditioning was given in four cases (3 related SCT, 1 unrelated SCT) and consisted of cyclophosphamide 120 mg/kg and fractionated total body irradiation (TBI) six doses of 200 cGy over 3 consecutive days. **Non-myeloablative** conditioning was given in seven cases (5 related and 2 unrelated SCT). These patients received thiotepa 10 mg/kg I.V. in two doses on day -6, fludarabine 30 mg/m^2 I.V. and cyclophosphamide 50 mg/kg I.V. starting on day -4 for 3 consecutive days. In the case of unrelated donors 200 cGy TBI was added on day -1.
3. *Graft-versus-host-disease (GVHD) prophylaxis*. Patients conditioned with the myeloablative regimen were given cyclosporine A (3 mg/kg I.V. from day -1) and a short course of methotrexate (on days +1, +3, and +6, 15 mg/m^2 on day + 1, 10 mg/m^2 successively) as standard GVHD prophylaxis.

   Oral cyclosporine (3 mg/kg, starting on day -1) and oral mycophenolate mofetil (15 mg/kg, days 0-35 after the transplant) was the prophylactic protocol against GVHD in the group undergoing non-myeloablative conditioning.

   In the case of unrelated donors the myeloablative protocol included anti-lymphocyte globulin (ATG 10 mg/kg I.V. per day, starting on day – 4 for 4 days), whereas alemtuzumab (20 mg/m^2 I.V for 3 consecutive days, starting on day -4) was added to the non-myeloablative regimen.

   We started tapering down the dose of cyclosporine on day +60 and, in the absence of clinical signs of GVHD, discontinued this drug on day +180. Tapering schedules of immunosuppressive drugs were modified at the discretion of physicians in the case of evidence of GVHD.
4. *Blood specimens.* Peripheral blood samples were collected in EDTA Vacutainers (K2E), between 9 a.m. and 12 noon and processed within 1 h of collection.
5. *Two-colour staining*. The patients' peripheral blood leukocytes were stained as described in a previous study[18]. Ten microlitres each of two directly conjugated antibodies, CD5-FITC and CD19-PE, were added to 100 µl of whole blood in EDTA and incubated for 15 min in the dark at room temperature. Isotype control monoclonal antibodies were used to determine the levels of background fluorescence.

   We used a double platform based on the whole blood protocol plus red cell lysis with ammonium chloride (10 min at room temperature) and two phosphate-buffered saline washing steps by centrifugation (300 x g, 10 minutes).

Full blood cell counts were performed using an automated haematology blood analyser (ADVIA 120, Bayer, Tarrytown, USA). Sample EDTA Vacutainer (K2E) tubes and monoclonal antibodies for specimen staining were purchased from Becton Dickinson (Italy).

6. *Flow cytometry analysis*. Flow cytometry was performed using a FACScan flow cytometer (Becton Dickinson, BD Biosciences) equipped with a 488-nm argon laser. The instrument setting included linear amplification (1024 channels) of the forward- and side scatter signals (FS and SS) and logarithmic amplification (4 log decades) of the FL1 and FL2 signals. Compensation parameters for multiparametric analysis were adjusted at the start of sample acquisition. A total of 5,000 events were acquired in the lymphocyte gate. The immunological gating strategy used CD19 versus FS to define B lymphocytes, whereas the absolute count referred to the gate defined on the basis of morphological parameters (SS versus FS).
Data were acquired and analysed using CellQuest software (Becton Dickinson, BD Biosciences) .
For the assay, the threshold was set on a FS parameter to exclude debris. Sequential gating strategies were established to define the distinct $CD19^+$ blood lymphocyte subsets: $CD19^+CD5^-$, and $CD19^+CD5^+$.
A complete haemogram was obtained for all patients.

Results

We found that the mean absolute number of peripheral blood B lymphocytes was, as expected, very low soon after allogeneic SCT but increased during the follow-up. Indeed, by 2 years after transplantation, the absolute number of peripheral blood B lymphocytes was higher in the post-transplant patients (635/μL: range, 228-903) than in a group of normal, young adults (about 200/μL)[20]. Similarly, the percentage of B lymphocytes among all lymphocytes was very low early after allogeneic SCT but increased throughout the 2-year follow-up we performed.

In contrast, the mean percentage of B-1a cells among all peripheral blood B lymphocytes was higher soon after transplantation and decreased with time, while the absolute number of B-1a cells increased over approximately the first 18 months following allogeneic SCT; subsequently the B-1a cells decreased and the absolute number of these cells found at the end of the 2-year follow-up was similar to that observed in a group of normal, young adults (about 40/μL)[20]. These results are summarised in Table I.

In spite of different haematological malignancies and different pre-transplantation treatments and conditioning regimens, B-1a cells were assessed by cytometry at various times also in 88 peripheral blood samples from 63 patients (42 males and 21 females; male:female ratio 2:1) who underwent SCT in the Bone Marrow Transplant Unit of the

Table I - Total B lymphocyte and B-1a cell counts over time after allogeneic stem cell transplantation

	n° CA		WBC/μL	Ly/μL	B cells/μL	%B-1a cells	B-1acells/μL
30 days	11	Mean	4424.5	857.2	1.76	24.2	0.28
		±SD	1989.7	413.1	1.39	28.3	0.34
90 days	11	mean	3710	959.1	55.2	29.6	18.3
		±SD	1131.9	302.4	12.9	26.0	31.2
180 days	11	mean	4399.1	1573.4	144	23.0	45.5
		±SD	1888.9	1358.7	132.9	21.0	65.0
360 days	9	mean	6337.5	2389.4	369.6	18.1	94.6
		±SD	1346.6	1311.6	462.5	9.9	164.0
540 days	5	mean	6786	2922.4	612.9	19.6	147.3
		±SD	1346.6	1311.6	462.5	9.9	164.0
720 days	5	mean	6893.3	3228.3	635.0	8.7	42.3
		±SD	3538.9	1691.2	358.2	6.5	23.9

Legends: n°CA: number of cytometric analyses; WBC/μL: absolute number of white blood cells per microlitre; Ly/μL: total number of lymphocytes per microlitre; B cells/μL: absolute number of B lymphocytes per microlitre; %B-1a cells: percentage of CD19/CD5 double positive cells among CD19 cells; B-1a cells/μL: absolute number of B-1a cells per microlitre.

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Haematology Section of Verona University. Thirty-nine samples were from patients who underwent to allogeneic BMT and 49 from patients who underwent peripheral blood SCT.

Cytometric analyses were performed within the first 100 days after transplantation (5 cases of BMT and 18 cases of peripheral blood SCT), after 100 days and within 1 year after transplantation (4 cases of allogeneic BMT and 17 cases of peripheral blood SCT), after 1 year and within 2 years after transplantation (7 cases of allogeneic BMT and 9 cases of peripheral blood SCT) and more than 2 years after transplantation (23 cases of allogeneic BMT and 5 cases of peripheral blood SCT).

Based on our data, the mean percentage of B-1a cells among the total peripheral blood B lymphocytes was higher soon after (<100 days) allogeneic peripheral blood SCT than after allogeneic BMT (41.3%, range 0-82% and 20.5%, range 0-66.7%, respectively). Subsequently, the mean percentage decreased in both groups and was similar after 2 years of follow-up (about 10%).

Conversely, the mean absolute number of B-1a cells was similar in the first year after allogeneic BMT and allogeneic peripheral blood SCT (101.7/μL, range 3.5-271/μL and 80.2/μL, range 0.9-357.3/μL respectively), while it was higher within the first 2 years after BMT than after peripheral blood SCT (99.8/μL, range 3.5-266/μL and 48.6/μL, range 1.1-190.8/μL respectively). However, the mean absolute number of B-1a cells in both groups was similar after 2 years of follow-up (about 50/μL).

Discussion

In mice, the generation of B-1 cells is largely confined to the foetal and neonatal periods, during which progenitors are initially present in the liver and can later be found in the spleen and bone marrow[10]. Very few B-1a cells develop from progenitors in adult bone marrow[21] and the reconstitution of B-1a cells in irradiated mice has been reported to be more efficient when the source of precursors is liver from foetal or neonatal mice rather than bone marrow[22].

In humans, very high percentages of B-1a cells are present in the foetal circulation, diminish in umbilical cord blood[23], and decrease in peripheral blood with age[20]. In adults, B-1a cells constitute about one-fifth of normal peripheral blood B lymphocytes[5]. Data regarding the reconstitution of the B-1a subset after allogeneic transplantation in humans are scarce.

Antin *et al*.[19] reported the reconstitution of peripheral blood B lymphocytes, also distinguished by the expression of Leu-1 (CD5), in patients who underwent allogeneic BMT for a heterogeneous group of disorders. Forty-six patients were studied by flow cytometric analysis at various times after transplantation and 20 of the 46 patients were followed serially. In six of these 20 patients the appearance of B-1a cells was reported to precede that of conventional B-2 cells by 1-2 weeks and in the remaining 14 patients the two types of cells appeared simultaneously. The two types of B cells were described to be present in equal numbers 1 month after allogeneic BMT and to persist at high levels for at least 1 year with a tendency for the number of B-1a cells to decline thereafter.

Conversely, according to our data, the recovery of B-1a cells after allogeneic SCT seems to be slower than reconstitution of the B-2 cell compartment, since B-1a cells were undetectable in 5/11 (45%), 3/11 (27%) and 2/11 (18%) patients in the first, third and sixth months after transplantation, respectively. The differences might be due to technical reasons; however to avoid analytical variability due to other cells or debris in the early post-transplantation phase, we used a CD45/CD14 control tube in the samples in which the lymphocyte gating by the morphological parameters (SS vs FS) was not pure.

It may be argued that marrow grafts are richer in B-1a cell precursors, the recovery of B-1a subsets proving more efficient after allogeneic BMT. In spite of this, the absolute numbers of B-1a cells per microlitre at the end of the reported follow-up were similar after both allogeneic BMT and peripheral blood SCT, and were also similar to the absolute number found in normal subjects of the same age group[20].

The reconstitution of the B-1a cell compartment after transplantation would, therefore, appear not to depend on the source of stem cells, possibly indicating that B-1a cell precursors are present in both marrow and peripheral blood and retain the capacity to replicate. Moreover, no substantial difference in the behaviour of B-1a cell reconstitution after myeloablative or non-myloablative regimens was noted.

The reported data shed no light on the debate regarding the origin of B-2 and B-1a cells., i.e. whether they derive from the same or different precursors. In spite of this, we may assume that B-2 and B-1a cell lineages are closely related and that the behaviour of B-1a cells after transplantation resembles that observed in humans with ageing.

The total percentage of B-1a cells is high early after transplantation and progressively decreases with time, while the absolute number increases over approximately the first 18 months after allogeneic transplantation only to decrease thereafter.

Further studies are necessary in order to clarify the origin of B-1a cells in humans in health and illness.

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