Acta Medica Okayama

Changes of lymphocyte subsets in leukemia patients who received allogenic bone marrow transplantation.

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Abstract

Proportional changes of lymphocyte subsets in the peripheral blood were monitored by two-color flow-cytometry in seven leukemia patients who had received allogenic bone marrow transplantation (BMT). Lymphocyte counts, and proportions of T and B-cells returned to normal ranges between the 2nd and 12th months after BMT. Activated T-cells prominently increased after BMT, and the values gradually returned toward normal. As to lymphocyte subsets, the proportions of CD 4+ cells had remained low, while those of CD 8+ cells high for a whole observation period after BMT. The changes of CD 4+ cells were caused by the decrease of suppressor-inducer T-cells (CD 4+ Leu 8+). High proportion of CD 8+ cells was mainly associated with increased suppressor T-cells (CD 8+ CD 11+). Among natural killer (NK) cells, highly active NK cells (CD 16+ CD 57-) markedly increased shortly after BMT, and gradually returned to normal. CD 16 -CD 57+ NK cells increased beyond normal ranges after the 2nd month. The incidence or degree of acute and chronic graft-versus-host diseases (GVHD) did not correlate with the changes of any lymphocyte subsets. The present results suggest that the increase of activated T-cells shortly after BMT reflects lymphocyte reconstitution. The prolonged immune deficiency after BMT might be related to either deficient expression of homing receptor (Leu 8 antigen) on CD 4+ cells or increased suppressor T-cells (CD 8+ CD 11+). In addition, the early increase of NK cells after BMT may compensate for the immune deficiency in BMT patients.

Changes of Lymphocyte Subsets in Leukemia Patients Who Received Allogenic Bone Marrow Transplantation

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Proportional changes of lymphocyte subsets in the peripheral blood were monitored by two-color flowcytometry in seven leukemia patients who had received allogenic bone marrow transplantation (BMT). Lymphocyte counts, and proportions of T and B-cells returned to normal ranges between the 2nd and 12th months after BMT. Activated T-cells prominently increased after BMT, and the values gradually returned toward normal. As to lymphocyte subsets, the proportions of CD 4$^+$ cells had remained low, while those of CD 8$^+$ cells high for a whole observation period after BMT. The changes of CD 4$^+$ cells were caused by the decrease of suppressor-inducer T-cells (CD 4$^+$ Leu 8$^+$). High proportion of CD 8$^+$ cells was mainly associated with increased suppressor T-cells (CD 8$^+$ CD 11$^+$). Among natural killer (NK) cells, highly active NK cells (CD 16$^+$ CD 57$^-$) markedly increased shortly after BMT, and gradually returned to normal. CD 16$^-$ CD 57$^+$ NK cells increased beyond normal ranges after the 2nd month. The incidence or degree of acute and chronic graft-versus-host diseases (GVHD) did not correlate with the changes of any lymphocyte subsets. The present results suggest that the increase of activated T-cells shortly after BMT reflects lymphocyte reconstitution. The prolonged immune deficiency after BMT might be related to either deficient expression of homing receptor (Leu 8 antigen) on CD 4$^+$ cells or increased suppressor T-cells (CD 8$^+$ CD 11$^+$). In addition, the early increase of NK cells after BMT may compensate for the immune deficiency in BMT patients.

Key words : immunologic reconstitution, lymphocyte subsets, graft-versus-host diseases, allogenic bone marrow transplantation

Major problems after allogenic bone marrow transplantation (allo-BMT) are infections due to prolonged immune deficiency and acute or chronic graft-versus-host diseases (GVHD). The exact mechanism of immune deficiency and GVHD after allo-BMT is still obscure, although several studies have suggested possible roles of T-cells and abnormal thymic functions (1, 2). Recently, it has become possible to identify various lymphocyte subsets with different functions using monoclonal antibodies (MoAb) to cell-surface antigens. To clarify the correlations of lymphocyte subsets with acute or chronic GVHD and complications after BMT, seven leukemia patients, who had received allo-BMT, were seri-

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ally monitored for lymphocyte subpopulations and their subsets by two-color flowcytometry using a number of MoAb. Lymphocyte proliferative responses to mitogens and levels of serum immunoglobulins and complement components were also determined.

Materials and Methods

Patients. Patients were 5 males and 2 females. Their mean age, when allo-BMT was performed, was 24.3 ± 6.2 years old (mean $\pm$ one standard deviation, range 15–31). Further details of the patients are shown in Table 1.

Allo-BMT was performed by using the following two conditioning regimens : (A) total body irradiation (TBI) 1200 rads, cyclophosphamide 60 mg/kg/day x 2 days, and ranimustine 200 mg, or (B) TBI 1200 rads, cytarabine 6 g/m² x 5 days, and etoposide 50 mg/kg. Patients were treated in bio-clean rooms during the first and/or second months after BMT. Mean age of HLA-identical donors to patients was 24.5 ± 10.6 (13–40) years old. Mean numbers of bone marrow cells transplanted to the patients were 3.7 ± 0.7 $(2.8 - 4.6)$ x 10⁸cells/kg.

Cyclosporin A, 3 mg/kg/day, was started in all the patients from one day before BMT for prevention of acute GVHD. After BMT, all the patients also received methotrexate, 15–19 mg (2nd day) and 10–13 mg (4, 7 and 11 th days), and intravenous gammaglobulin, 10–15 g x 2 days/2 weeks for 3 months. Five of the patients received recombinant human granulocyte-colony-stimulating factor, 250 μg/body, from the 6th day after BMT. Dosages of these medications were reduced and then stopped. Prednisolone, 30–60 mg/day, was started, if necessary, for acute or chronic GVHD and other complications.

Acute GVHD was graded from Grades 0 to IV according to the method previously reported (3). Acute GVHD was observed in four (57%) of the patients, and two patients developed chronic GVHD with mucositis and skin involvements. Three of the 7 patients developed pleuritis with or without pericarditis of unknown causes after BMT (6, 7 and 8th months, respectively). One patient developed cytomegalovirus (CMV) -associated acute interstitial pneumonia 7 months after BMT, and died in the 8th month. Acute leukemia relapsed in another patient 7 months after BMT. The mean follow-up period of 7 patients after BMT was 19.4 ± 12.8 (range : 7.5–37) months. Lymphocyte subsets were monitored for 3.5–20 (10.1 ± 5.4) months after BMT.

MoAb. OK-series and Leu-series MoAb were pur-

Tabel 1 Patient's profiles.

Clinical conditions	Case number						
	1	2	3	4	5	6	7
Recipients							
Sex	M	M	M	M	M	F	F
Age (years old)	22	18	15	26	31	31	27
Basic disease	CML	CML	AML	AML	CML	ALL	ALL
Conditioning regimens	A	A	B	B	B	B	B
Observation period (months)	37	32	29	8	13	9	7.5
GVHD Acute	II	II	0	III	0	0	III
Chronic	−	+	+	−	−	−	−
Complications	P	P	P/PC	CMV-IP	−	ALL-relapse	−
Donors							
Sex	M	F	F	F	M	M	M
Age (years old)	20	13	17	38	40	20	30
Transplanted Cells (x10⁸cells/kg)	3.0	2.8	4.3	4.1	3.6	3.2	4.6

Abbreviations : CML = chronic myeloid leukemia, AML = acute myeloid leukemia, ALL = acute lymphoblastic leukemia, GVHD = graft-verus-host disease, P = pleuritis, P/CP = pleuritis and pericarditis, CMV-IP = cytomegalovirus-associated interstitial pneumonia. Bone marrow transplantation (BMT) was performed at the time of chronic phase of CML, the 2nd complete remission of AML or ALL. Two conditioning regimens, A and B, were employed (see the text). All the donors are HLA-identical siblings of the patients.

chased from Ortho Diagnostic Systems Inc. and Becton-Dickinson Immunocytometry Systems(B/D), respectively. The following combinations of fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-labelled MoAb were employed : OKT 3 (CD 3)/OKB 7 (CD 21), OKT 3/OKDR, OKT 4 A (CD 4)/OKT 8 (CD 8), Leu 8/OKT 4, OKT 8/Leu 15 (CD 11 b), Leu 7 (CD 57)/Leu 11c (CD 16). A mixture of FITC- and PE-labelled murine IgG (Simultest control®, B/D) was used as a control reagent. Ten μl of OK-series MoAb and 20 μl of Leu-series MoAb were used for each test.

Two-color flowcytometry. The method was already reported elsewhere (4). Briefly, peripheral blood mononuclear cells (PBMC) were separated by the standard Ficoll gradient centrifugation. Erythrocytes in the PBMC were lysed with hemolytic solution. After washed with phosphate buffered saline (PBS), PBMC were suspended in PBS containing 5 % heat-inactivated fetal calf serum (FCS) and 0.1 % sodium azide. One hundred μl of PBMC (1×10^7 cells/ml) was mixed with FITC-MoAb and corresponding PE-MoAb, and incubated at 4°C for 30 min. After washed with 0.1 % sodium azide-PBS, cells were preserved in 1 % paraformaldehyde-PBS at 4 % for 16 h under light shielding. After passing cells through a nylon-mesh filter, five thousand cells were measured by a flowcytometer (FACSTAR®, B/D). Lymphoid cells, surrounded by appropriate foward and 90°C light scatter gates, were analyzed. Percentages of the cells positively or negatively stained with MoAb were determined by setting thresholds using control cells mixed with FITC-and PE-murine IgG : negative cells for both FITC-and PE-murine IgG were always more than 98 % in the controls.

Normal values of the lymphocyte counts and percentages of lymphocyte subsets were obtained from 34 healthy adults, 15 males and 19 females. Their mean age was 36.1 ± 13.6 years old.

Lymphocyte proliferative responses to mitogens. Proliferative responses of PBMC to phytohemagglutinin (PHA) or concanavalin A (Con A) were determined by the standard method using ^{3}H-thymidine (5). Briefly, PBMC (1×10^5cells/well) in 10 % FCS-RPMI 1640 medium were incubated in the presence or absence of each mitogen (the final concentration of PHA : 20 μg/ml, or Con A : 10 μg/ml) at 37°C for 64 h in 5% CO$_2$. 0.25 μCi of ^{3}H-thymidine was added to each well and further incubated for 8 h. Cells were harvested and thymidine incorporation was determined by liquid scintilation spectrophotometry.

Serum levels of immunoglobulins (IgG,IgA and IgM)

and complements (C 3 and C 4) were determined by using a laser nephelometer analyzer and antisera against each serum component (Behring).

Results

Peripheral blood (PB) neutrophil and lymphocyte counts. Neutrophil counts retured to normal ranges within 2 months after BMT in most patients (data not shown). Lymphocyte counts returned to normal ranges within 6 months in 3 patients and within 12 months in 3 other patients after BMT (Fig. 1). The counts were still low on the 13 th month after BMT in one patient.

B and T-cells. Percentages of mature B-cells (CD 21$^+$) returned to normal values within 2 months after BMT in 2 patients, but values remained below normal in 3 patients between the 6 and 20 th months (Fig. 1). B-cells markedly increased in case No. 3, when he developed pleuritis and pericarditis of unknown cause on the 8 and 9 th months after BMT. Percentages of mature T cells (CD 3$^+$) returned to normal values within 6 months in most patients, except 2 patients who were receiving prednisolone.
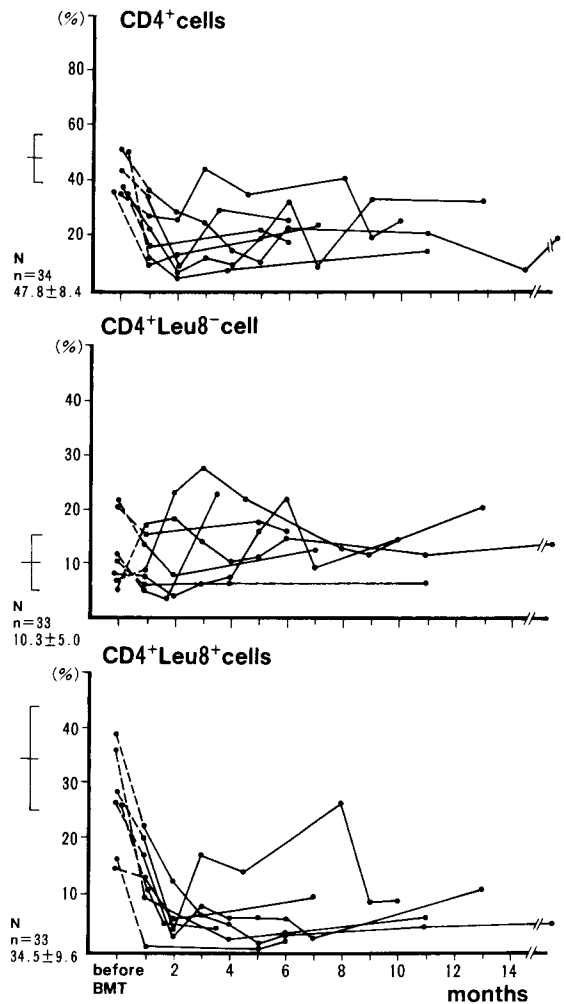
CD 4$^+$ cells and their subsets. Helper / suppressor-inducer T-cells (CD 4$^+$) remained clearly below normal values during the whole observation period after BMT (Fig. 2). The CD 4$^+$cells were further separated into two subsets, helper T-cells (CD 4$^+$Leu 8$^-$) and suppressor-inducer T-cells (CD 4$^+$Leu 8$^+$)(6, 7). Low levels of CD 4$^+$Leu 8$^+$ cells persisted for the whole observation period after BMT, while percentages of CD 4$^+$Leu 8$^-$ cells had remained at slightly elevated levels in most patients, as compared with values from healthy controls. Therefore, proportional changes of CD 4$^+$ cells after BMT were considered to be caused by the decrease of CD 4$^+$ Leu 8$^+$ cells.

CD 8$^+$ cells and their subsets. Percentages of CD 8$^+$ cells, which include suppressor / cytotoxic T-cells and some of the natural killer

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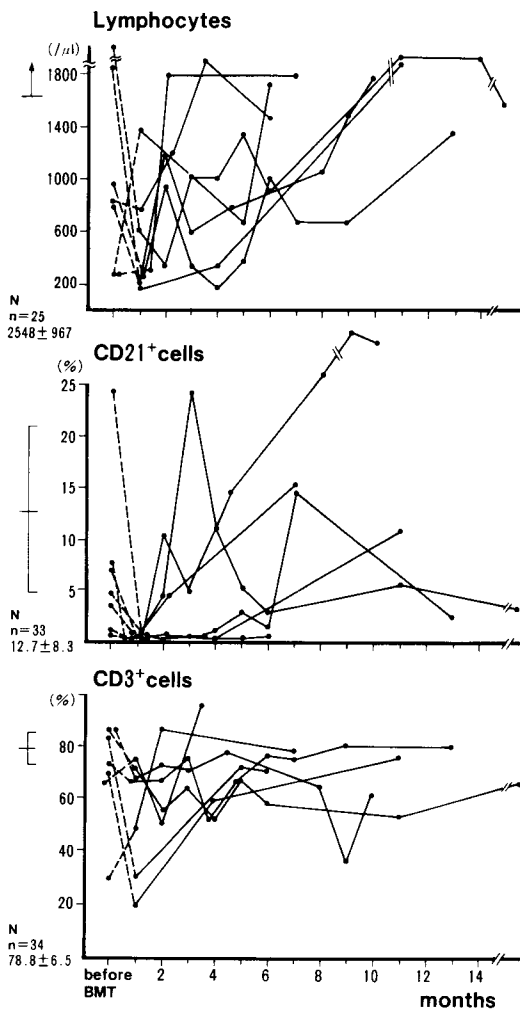
Fig. 1 Recovery of total lymphocyte counts, CD 21$^+$B cells and CD3$^+$T cells after allogenic bone marrow transplantation (BMT) : Changes of each parameter between before and one month after BMT are shown by dashed lines. Normal values (N, mean ± one standard deviation) obtained from healthy adults are indicated on the left side of figures.

Fig. 2 Proportional change of CD4$^+$ cells and their two subsets after BMT.

(NK) cells, returned to normal values shortly after BMT and the values had further increased above normal ranges in most patients (Fig. 3). CD 8$^+$ cells were also separated into two subsets, suppressor T-cells (CD8$^+$CD 11$^+$) and cytotoxic T-cells (CD 8$^+$CD 11$^-$)(8, 9). Proportions of CD 8$^+$CD 11$^+$ cells rapidly recovered and reached

levels higher than normal after BMT. These values remained elevated in most patients. Proportions of CD 8$^+$CD 11$^-$ cells also recovered shortly after BMT, but the levels remained within the normal range in most patients. High proportions of CD 8$^+$ cells after BMT, therefore, reflect the increase of CD 8$^+$CD 11$^+$ cells.

CD 4$^+$/CD 8$^+$ cell ratio and activated T-cells. According to the proportional changes of CD 4$^+$ or CD 8$^+$ cells described above, the ratio

of CD 4^+/CD 8^+ cells was markedly low after BMT, and the low values persisted throughout the observation period (Fig. 4). Proportions of activated T-cells (HLA-DR$^+$CD 3^+) in total CD 3^+ cells during the first three months after BMT were markedly increased as compared with normal values. The values returned very gradually toward normal.

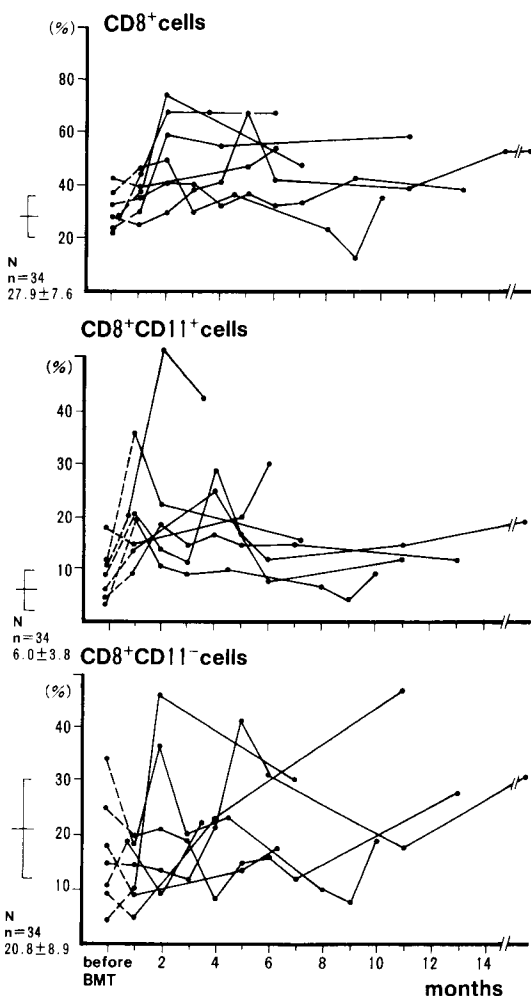*NK cells.* Three NK cell subsets were determined using CD 16 and CD 57 MoAb. Percent-
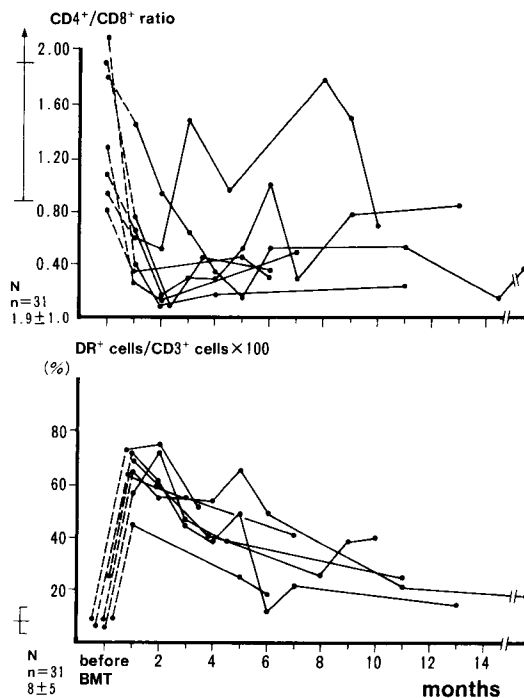

Fig. 4   Ratio of CD 4^+/CD 8^+ cells and proportion of activated (HLA-DR$^+$) cells in CD 3^+ cells.

ages of CD 16^+CD 57^- cells, which are reported to be a highly active NK cell subset (10), were markedly high shortly after BMT as compared with normal values, and the values gradually returned to normal (Fig. 5). Proportions of CD 16^+CD 57^+ cells with variable NK activity and those of CD 16^-CD 57^+ cells with weak NK activity also recovered shortly after BMT, while the CD 16^-CD 57^+ cells further increased between the 2nd and 6th months.

Associations between GVHD or other complications and changes of lymphocyte subsets after BMT. Acute or chronic GVHD were not associated with high proportions of activated T-cells. Two patients who had high proportions of cytotoxic T-cells did not develop acute GVHD, while the disease was observed in those who had normal or low proportions of the subset. In
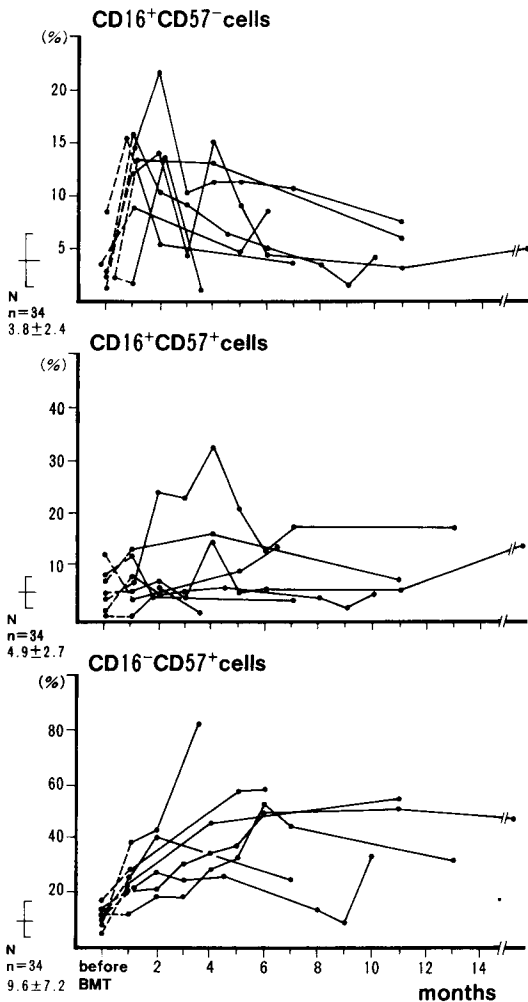

Fig. 3   Proportional change of CD 8^+ cells and their two subsets after BMT.

CD16⁺CD57⁻cells

CD16⁺CD57⁺cells

CD16⁻CD57⁺cells

Fig. 5   Proportional change of three natural killer cell subsets after BMT.

addition, high proportions of CD 16⁺CD 57⁺ cells were not associated with acute GVHD. Proportions of CD 16⁺CD 57⁻ and CD 16⁺CD 57⁺ cells became lower on the 4th month after BMT than normal values in case No. 4, and then he developed CMV-associated pneumonia on the 7th month after BMT. Other parameters of lymphocyte subsets, however, were not associated with acute and chronic GVHD or observed

complications.

Lymphocyte proliferative responses to mitogens. Lymphocyte proliferative responses to PHA or Con A decreased shortly after BMT, and gradually returned to the values before BMT (within 6 –10 months) (Fig 6).

Serum levels of immunoglobulins and complements. IgG levels remained within normal values after BMT, due to regular gammaglobulin injections (Fig. 7). After discontinuation of injections, IgG levels decreased transiently between the 4th and 6th months in some patients. IgA levels decreased after BMT, and the low levels had persisted for 6 months or more. IgM levels recovered 4 months after BMT, and the levels further increased after the 6th month in some patients, especially in the two patients who developed chronic GVHD (cases No. 2 and 3). Levels of serum C 3 and C 4 did not change after BMT in the 5 patients examined (data not shown).
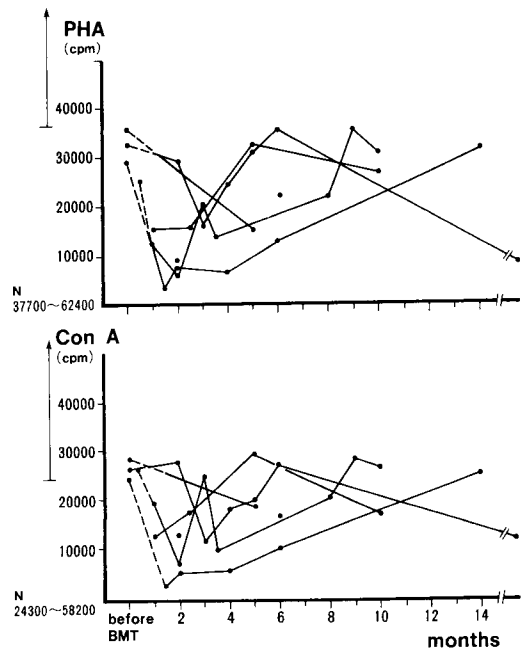
PHA (cpm)

Con A (cpm)

Fig. 6   Lymphocyte proliferative responses to phytohemagglutinin (PHA) and concanavalin A (Con A).
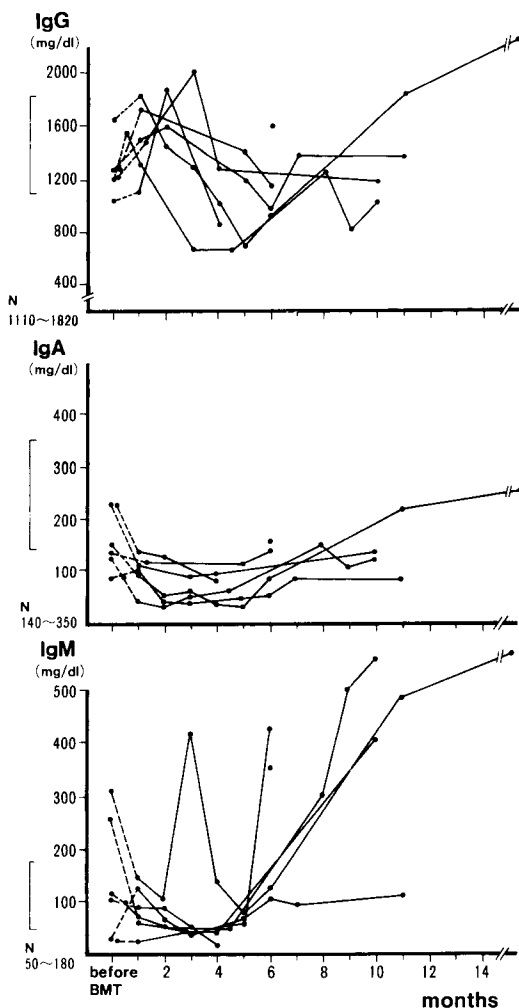
Fig. 7   Serum immunoglobulin levels after BMT.

Discussion

Acute GVHD usually develops between the first and 3rd months after allo-BMT, while chronic GVHD occurs after the 3rd month. Chances of casual infections probably persist for many years after BMT. Changes of T cells and their subsets, functional damages of the thymus and donor-recipient histo-incompatibility might be important for these events (1, 2).

In the present study, numbers of lymphocytes and percentages of T-cells and B-cells returned to the normal ranges between the 2nd and 12th months after BMT, although the recovery delayed in some patients, as compared with previous reports (1, 11, 12).

Activated T-cells prominently increased shortly after BMT, and remained at high levels up to 12 months or more after BMT. The result is compatible with a previous report by others (13). The increase of activated T-cell has been reported to reflect the onset of acute or chronic GVHD (14), although present observations did not confirm this. The present results, however, supported another possibility (13) that the increase of activated T-cells might simply reflect the process of lymphocyte reconstitution (proliferation) after BMT, but not lymphocyte responses to allogenic antigens. This is also supported by the evidence that an increase of activated cells was recognized in patients receiving autologous BMT which lacks GVHD (13).

After BMT, propotions of $CD4^+$ cells remained below normal in the present patients, while those of $CD8^+$ cells increased above normal ranges. These results were similar to those reported by others (11, 12, 15),

Subsets of CD 4^+ cells have been determined using CD 45 R (2 H 4) MoAb and 4 B 4 MoAb (13, 14). Leu 8 MoAb was first employed in the present study to determine helper and suppressor-inducer T-cells in patients who had received BMT. Although CD 45 R/4 B 4 and Leu 8 MoAb do not recognize identical subsets, present results demonstrated that suppressor-inducer T-cells (CD 4^+Leu 8^+) remained in below normal ranges for more than 12 months after BMT, as recognized in the previous studies using CD 45 R MoAb (13, 14). Helper T-cells (CD 4^+4 B 4^+) were reported to increase after BMT (14), but CD 4^+Leu 8^- helper T-cells remained at slightly high levels in the present patients. The antigen recognized by Leu 8 MoAb was recently identified as a homing receptor of lymphocytes into peripheral lymphoid organs (16). Snice migration of antigen-stimulated lymphocytes into

lymphoid organs will be important for normal immune responses, deficient expression of Leu 8 antigen on CD 4$^+$ cells, as observed in this study, might be responsible for prolonged immune deficiency in BMT patients.

Subsets of CD 8$^+$ cells have also been examined using the same MoAb (CD11b) employed in this study (13, 14, 17). The present study demonstrated an increase of CD 8$^+$CD 11$^+$ cells for more than 12 months after BMT, as observed in previous reports (13, 17). The increased CD 8$^+$CD 11$^+$ suppressor T-cells might be important for immune deficiency in BMT patients. In addition, present results and those reported by others (17) demonstrate that two subsets of CD 8$^+$ cells were not associated with acute or chronic GVHD. However, another report identified an increase of CD 8$^+$CD 11$^-$ cells after BMT, and the cells were associated with chronic GVHD (14). The reason for these discrepancies is currently unknown.

NK cell activity after BMT has been reported to recover within 2 months after BMT (1, 18, 19), while NK cell numbers were only examined in a previous study (13) ; an early and transient increase of CD 16$^+$ cells after allo-BMT (within 3 months) is recognized. The present study using two MoAb against NK cells further clarified that the early increase of CD 16$^+$NK cells was caused by an increase of highly active NK cells (CD 16$^+$ CD 57$^-$), but not CD 16$^+$CD 57$^+$ cells, while CD 16$^-$CD 57$^+$ cells gradually increased after BMT. However, the changes of NK cells after BMT did not correlate with the development or degree of GVHD. One patient who developed CMV-associated pneumonia following the decrease of active NK cell subsets suggests that an increase of NK cells might complement deficiency of T-cell-mediated immune defense after BMT.

The recovery of other immunological parameters after BMT, such as lymphocyte proliferative responses to mitogens, and levels of serum IgG, IgA and IgM, was similar to previous reports (1, 11, 12, 15, 20), except for IgG level which had been maintained by repeated intravenous gam-

maglobulin injections. Persistent low levels of IgA after BMT were considered to indicate deficient B-cell differentiation in the patients. Furthermore, present results suggest an association of high IgM levels with chronic GVHD.

Finally, we should also consider the possibility that delayed recovery of several immunologic parameters observed in some of our patients is, at least partially, caused by immunosuppresive therapy with cyclosporin A, methotrexate or prednisolone, which was used for prevention and treatment of GVHD or other complications.

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