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Reconstruction of the Immune System after Unrelated or Partially Matched T-Cell-Depleted Bone Marrow Transplantation in Children: Functional Analyses of Lymphocytes and Correlation with Immunophenotypic Recovery following Transplantation

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Reconstitution of the immune system following T-cell-depleted bone marrow transplantation (BMT) in children has yet to be fully elucidated. Thus, we prospectively studied the recovery of immune function in 64 children who underwent T-lymphocyte-depleted marrow transplants using either matched family member donors or matched unrelated donors. We measured in vitro posttransplantation proliferative responses to phytohemagglutinin (PHA), concanavalin A, pokeweed mitogen, and *Candida albicans* antigen and assessed unidirectional allogeneic mixed-lymphocyte culture (MLC) responses at various times. A total of 129 healthy individuals served as normal controls for these assays. Responses to T-cell mitogens normalized within 12 months posttransplantation, while MLC responses normalized by 9 months. The presence of graft-versus-host disease (grade II or greater) and cytomegalovirus infection was associated with delays in immune function recovery. Importantly, immune function recovery correlated temporally with a rise in peripheral lymphocyte count. In contrast, the CD4/CD8 ratio was not predictive of immune recovery. Knowledge of immune function recovery may guide clinicians in devising strategies to minimize the risk of infection post-BMT.

Alternative sources of hematopoietic stem cells for allogeneic bone marrow transplantation (BMT) have been used for children lacking histocompatible related donors. Marrow transplantation using either closely matched unrelated donors (UD) or partially matched family donors (FD) has been done in many programs throughout the world (9, 30). Intensive conditioning regimens to facilitate engraftment and increased graft-versus-host disease (GvHD) prophylaxis have been applied to these transplants, including depletion of T lymphocytes with or without additional immunosuppression posttransplantation (31, 36). Due to the aggressive immunosuppression post-marrow transplantation required for successful engraftment, recipients of unrelated donor or HLA-nonidentical transplants appear to have a higher rate of infectious complications than those of transplants using matched sibling donors (11, 27, 30).

The proliferative response of lymphocytes to mitogens is commonly used to assess immune recovery after transplants. Analyses of immune recovery following autologous or matched-sibling BMTs in adults have indicated that it takes a minimum of 6 months to more than a year to achieve normalization of immunologic responses (1, 40). However, only a few reports of immune recovery in children following BMT have been described (6, 10, 15, 16). Our study is unique in describing immune functional reconstitution exclusively after unrelated or partially matched, T-cell-depleted BMT in children. We analyzed further various patient characteristics, including age, marrow cell dose, donor type (UD or FD), presence of GvHD, and cytomegalovirus (CMV) status to determine their predictive value relative to recovery of in vitro immune responses. Because functional studies are costly and time-consuming, we also analyzed the data to assess whether immunophenotyping alone could predict immune function recovery. The understanding of immune reconstitution post-BMT may eventually help provide better strategies to accelerate immune recovery and prevent infectious complications inherent in this particular transplant setting.

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MATERIALS AND METHODS

Patient population. Two hundred three children underwent allogeneic BMT using UD or FD at the University of Iowa Hospitals and Clinics from June 1986 to August 1993. The following patients were excluded from analysis: patients surviving less than 3 months ($n = 63$), patients who received transplants after June 1993 ($n = 8$), patients who did not achieve successful lymphohematopoietic engraftment ($n = 25$), patients who received multiple transplants, or those who received initial transplants at other institutions ($n = 28$). Immune function was analyzed prospectively at 1, 2, 3, 6, 9, 12, 18, 24, and 36 months post-BMT. An initial analysis of these data in 1989 found poor immune function during the first 3 months following transplantation. Subsequently, immune function analyses were initiated 6 months posttransplantation.

A total of 64 patients with various diseases remained eligible for the study. Patient characteristics are shown in Table 1. Fifty-four patients received marrow from FD (mismatched in one to three HLA antigens) and 10 received marrow from UD. Fifty-six percent of patients had no evidence of significant GvHD; 44% developed a grade II or greater acute GvHD, chronic GvHD, or both. CMV positivity was defined as isolation of virus from any culture source and/or a CMV immunoglobulin M (IgM) response of >0.6 as determined by microparticle enzyme immunoassay posttransplantation. CMV IgG titers were not assessed because of routine intravenous immune globulin infusions posttransplantation.

TABLE 1. Patient eligibility and characteristics

Characteristic[a]	Values for group
No. of eligible patients	
Total	64
ALL	33
ANLL	12
CML (JCML)	11 (4)
SAA	3
Inborn errors of metabolism	4
Wiscott-Aldrich syndrome	(1)
Hurler syndrome	(2)
Metachromatic leukodystrophy	(1)
Non-Hodgkin's lymphoma	1
Age (yr)	
Mean ± SD	7.8 ± 4.4
Median	7.0
Range	1.1–18.4
No. of patients	
<5	20
≥5 and <10	24
≥10	20
Cell dose[b] (10^8/kg of recipient's wt)	
Mean ± SD	3.7 ± 1.8
Median	3.3
Range	0.9–9.1
No. of patients with the following no. of donors:	
<3	26
≥3	38
Types of donors	
Partially matched family	54
Closely matched unrelated	10
GvHD	
None	36
Grade II or more	28
CMV status[c]	
Negative	49
Positive	15

[a] ALL, acute lymphocytic leukemia; ANLL, acute nonlymphocytic leukemia; CML, chronic myelogenous leukemia; JCML, juvenile chronic myelogenous leukemia; SAA, severe aplastic leukemia.

[b] Nucleated cells post-T-cell depletion, uncorrected for peripheral blood leukocyte count.

[c] CMV positivity was defined by a positive CMV IgM response posttransplantation or the presence of positive systemic CMV culture (e.g., urine, blood, or other body fluid). Patients had to be alive at 3 months post-BMT to be included in these studies.

Fifteen patients were classified as positive for CMV. All patients were treated according to protocols approved by the Human Subjects Committee at the University of Iowa Hospitals and Clinics and The University of Iowa College of Medicine, and informed written consent was obtained.

Patient management. All patients were cared for in the Pediatric Bone Marrow Transplant Unit. Detailed protocols of patient management have been described elsewhere (15). The preparative regimen included etoposide, cytosine arabinoside (ara-C), cyclophosphamide, and intrathecal ara-C. Total body irradiation was administered at 1,200 cGy in six fractions. Bone marrow was depleted of T lymphocytes by using CT-2, an anti-CD2 IgM mouse monoclonal antibody, and rabbit serum as a source of complement (31, 32). In addition, all patients received anti-lymphocyte globulin and prednisone as GvHD prophylaxis. Cyclosporine was reserved for patients with acute and chronic GvHD who were unresponsive to steroids.

Immune function analyses. (i) Proliferative responses. After Ficoll-Hypaque density sedimentation, peripheral blood mononuclear cells were resuspended in RPMI 1640 culture medium supplemented with 20% normal human serum and 100 μM glutamine for proliferation assays. The cells were plated in triplicate in round-bottom, 96-well microtiter trays at 5×10^4 per well with the following optimal concentrations of mitogens: phytohemagglutinin (PHA) (Gibco, Grand Island, N.Y.), 1, 5, and 10 μg/ml; concanavalin A (ConA) (Sigma, St. Louis, Mo.), 5 μl of a 0.2% stock solution per well; pokeweed mitogen (PWM) (Gibco), 5 μl of rehydrated stock solution per well; tetanus toxoid (TT) (Wyeth, Marietta, Pa.), 5 μl of a 1:50 working stock solution per well; and *Candida albicans* (Hollister Stier, Elkhart, Ind.), 5 μl of a 1:30 (wt/vol) stock solution per well. Cells were cultured with PHA and ConA for 4 days, with PWM for 5 days, and with TT and *C. albicans* for 6 days at 37°C in a humidified, water-jacketed incubator in a 5% CO_2 atmosphere. During the last 6 h of culture, cells were labeled with 1 μCi of [^{3}H]thymidine (6.8 μCi/mM) (New England Nuclear, Boston, Mass.) per well prior to harvest. Cultures were processed with an automated microplate harvester (Cambridge Technology, Cambridge, Mass.), and incorporation of [^{3}H]thymidine was measured in a liquid scintillation counter.

(ii) MLC. Unidirectional allogeneic mixed-lymphocyte cultures (MLC) were performed in triplicate in round-bottom plates (Costar, Cambridge, Mass.) with 5×10^4 responders and an equal number of irradiated (21 Gy) stimulator cells in 0.2 ml of RPMI 1640 culture medium. After incubation for 7 days, the cultures were pulsed with [^{3}H]thymidine as described above. Data are expressed as the mean counts per minute of triplicate cultures ± standard deviations.

Statistical analyses. Proliferative responses and MLC results were interpreted by two methods. The first was to calculate a relative response (RR) as a percentage of the mean of two normal control responses studied on the same day (22). Results above 30% RR were considered normal (4). The stimulation index (SI) was expressed as follows: SI = (cpm for test − cpm for unstimulated cell control)/cpm for unstimulated cell control (14). One hundred twenty-nine healthy individuals served as normal controls for the SI. Immune function results were considered normal when the mean of each SI became greater than the fifth percentile for the control population.

The mean ± standard deviation was used as a quantitative descriptor of distribution. The RR to and SI for the respective mitogen and MLC were aggregated for each patient over intervals of 1, 2, 3, 6, 9, 12, 18, 24, and 36 months. Patient characteristics were analyzed as variables with respect to mitogen and MLC results. Mean values were compared to detect significant differences by the Mann-Whitney rank-sum test for two subgroups (e.g., CMV-positive and -negative groups). Likewise, Kruskal-Wallis analysis of variance was used when more than two groups were compared (e.g., the three age subgroups). Statistical analyses were performed with the Biomedical Data Processing (BMDP) statistical package (BMDP Statistical Software, Inc., Los Angeles, Calif.).

Correlation between immunophenotype and immune function. Immunophenotypic variables (total lymphocyte number, mature T-cell [CD3] number, helper T-cell [CD4] number, B-cell [CD20] number, NK cell [CD16/CD56] percentage, and CD4/CD8 ratio) were analyzed with respect to functional data (mitogen responses to PHA, ConA, and PWM and MLC response to alloantigens). The number of patients analyzed at each point were 16 for 1 month, 18 for 2 months, 17 for 3 months, 52 for 6 months, 28 for 9 months, 31 for 12 months, 23 for 18 months, 23 for 24 months, and 17 for 36 months.

Each immunophenotypic result was plotted against respective matched function data. Individual correlation was calculated for patients having more than four matched pairs of data. Data for 20 to 25 patients were used in this analysis. The correlation coefficient (r) estimating the degree of closeness of linear relationship between the immunophenotypic results and the immune function results was calculated. If the null hypothesis that the patients' correlation coefficients are estimates of the same correlation coefficient was not rejected ($P > 0.10$), the patients' correlation coefficients were combined to obtain an estimate of the common values and the 95% confidence limits (28). When r is >0.5, >25% of the immunophenotypic variables can be attributed to their linear regression on the immune function data, implying a satisfactory correlation.

RESULTS

Proliferative response to mitogens, specific antigens, and MLC. The function of peripheral blood lymphocytes from recipients of T-cell-depleted marrow transplants was tested in proliferation assays by stimulation with the T- and B-cell mitogens, specific antigens, and MLC responses to third-party alloantigen (Fig. 1 and 2). Results are expressed as relative responses or stimulation indices. Table 2 summarizes these data, demonstrating that responses to PHA and ConA and the B-cell mitogen PWM were depressed until 12 months posttransplantation. Unidirectional MLC responses to alloantigen were low during the first 3 months and normalized by 9 months posttransplantation.

The T-cell responses to specific antigens, *C. albicans* antigen and TT, were also depressed after transplant. The SIs failed to reach normal values by 36 months posttransplantation. However, analysis of the RR to *C. albicans* was found to be >30% of the normal value by 9 months, while the RR to TT normalized by 24 months posttransplantation.

Various patient characteristics were analyzed to find correlations with the recovery of immune function. The effects of age, presence of GvHD, and CMV status are summarized in

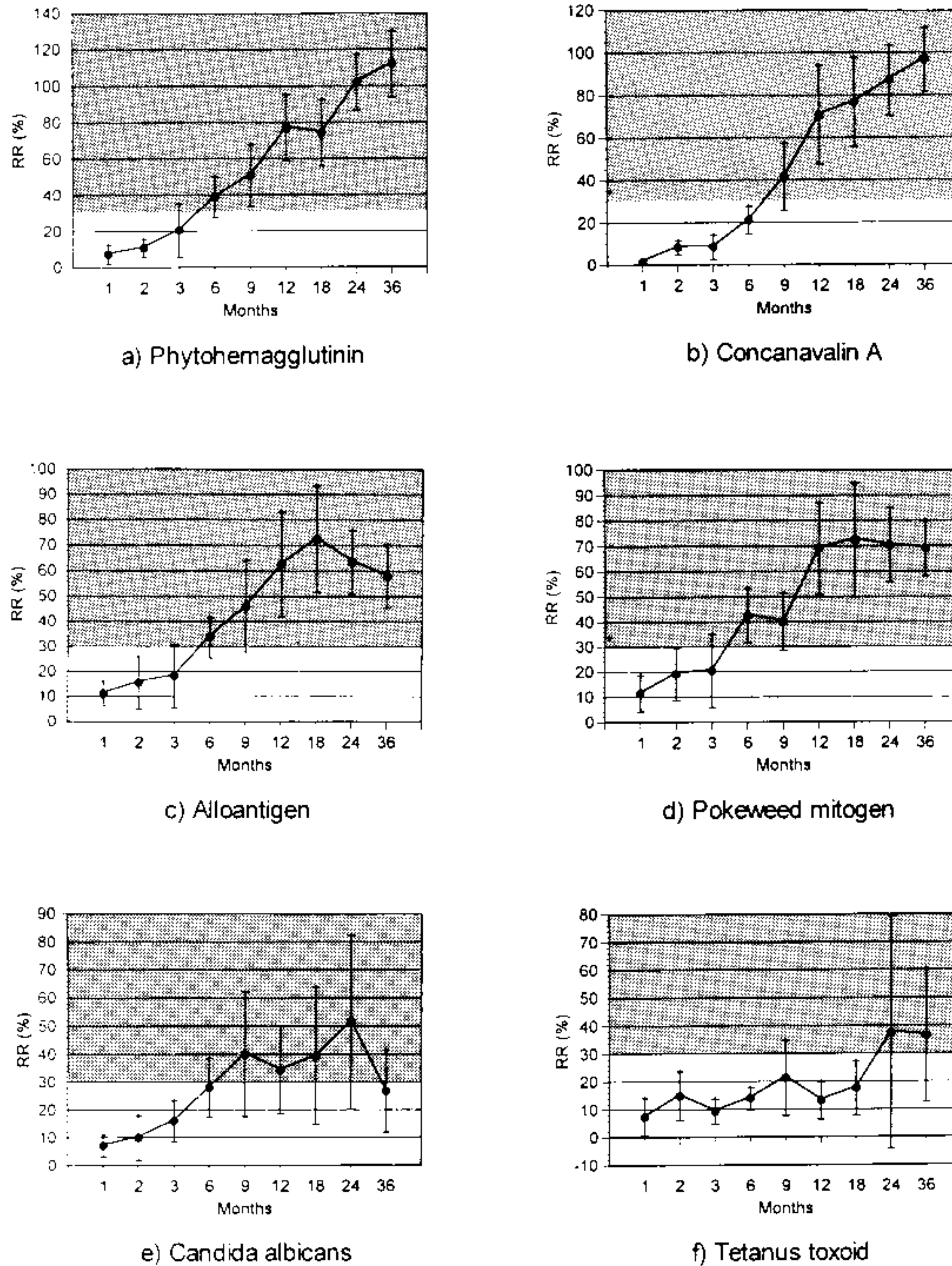

FIG. 1. Proliferative responses to mitogens and MLC response to alloantigen expressed as RR. The results above 30% RR were considered normal (shaded areas).

Table 3. Younger patients tended to have higher proliferative responses to T-cell mitogens than their older counterparts. Patients having acute grade II or greater GvHD and/or chronic GvHD had significantly diminished proliferative responses to T-cell mitogens (Fig. 3). Most differences were noted between 9 and 24 months after transplantation. Patients with CMV positivity showed significant delay in recovery of proliferative responses to mitogens compared to CMV-negative patients, especially at 6 to 9 months following transplantation (Table 3; Fig. 4). In contrast, MLC reactivity and responses to specific antigens were not affected by any patient characteristics in this study. Nucleated-cell dose did not influence the rate of immune recovery. In addition, 84% of the children were transplanted with marrow from partially matched family members; thus, insufficient information (in terms of patient numbers) was available for transplants using UD to determine the impact of donor type on the rate of immune recovery.

Comparison and correlation between immunophenotypic and immune function data. The kinetics of CD4/CD8 ratio recovery were compared with proliferative responses to PHA.

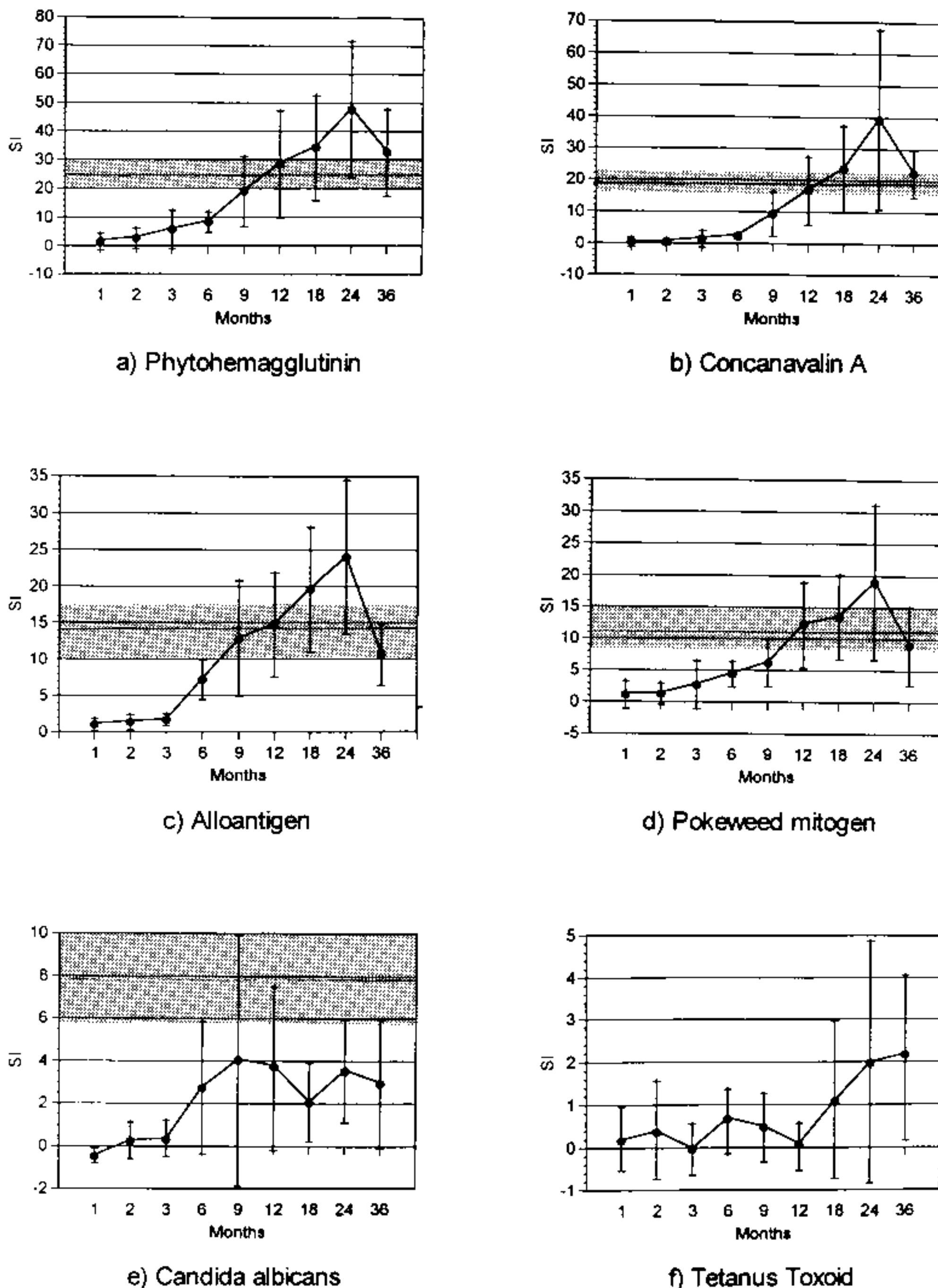

FIG. 2. Proliferative responses to mitogens and MLC response to alloantigen expressed as SI. Fifth and 95th percentiles for the normal population were defined as the shaded area with mean. The SI for TT remained far below the normal ranges throughout the study period.

The CD4/CD8 ratio was considered normal when the value was greater than the fifth percentile for healthy children (8). Normalization of proliferative responses preceded CD4/CD8 ratio recovery in 14 patients, while 19 patients had an earlier recovery of CD4/CD8 ratio. Three patients had simultaneous normalization of both parameters. This analysis could not be completed for 28 patients, as immune recovery was incomplete by 36 months after transplantation.

The correlation between six different immunophenotypic parameters and four immune function analyses was monitored (Table 4). The total lymphocyte count had good correlation ($r > 0.5$) with proliferative responses to PHA, ConA, and PWM. $CD3^+$ cell number correlated well with responses to PHA and the MLC response to alloantigen, suggesting that immunophenotypic recovery might predict return of T-cell function. Although NK cell percentage inversely correlated with recovery of T-cell function, only a minor portion of the variation in the immune function analyses could be attributed to their linear regression on immunophenotypic parameters ($r < 0.5$). The recovery of $CD20^+$ cell number and CD4/CD8

TABLE 2. Time to normalization of immune responses

Stimulus	RR (mo)	SI (mo)
PHA		
1 μg/ml	6	18
5 μg/ml	6	12
10 μg/ml	6	12
ConA	9	12
Alloantigen	6	9
PWM	6	12
Streptokinase	Never normal	Never normal
TT	24	Never normal
C. albicans	9	Never normal

ratio were unable to predict the recovery of any of the immune function parameters, as a common correlation coefficient could not be estimated ($P > 0.10$).

DISCUSSION

Reconstitution of the immune system following BMT is characterized by a recapitulation of normal lymphoid ontogeny. Immune recovery is known to be affected by GvHD and/or the necessary immunosuppressive therapy used to treat or prevent GvHD (1, 20, 21, 42). Other factors influencing immunological reconstitution include the underlying disease for which the BMT was performed, the donor-recipient relationship, the degree of histocompatibility or histoincompatibility, the time to achieve engraftment, and concurrent infections (11, 21, 30). Although immune reconstitution following BMT has been widely documented in adults (1, 40), little is known about the immune recovery in children following T-cell-depleted BMT from less than perfectly matched sibling donors. Thus, we prospectively studied the reconstitution of immune function in this patient population by in vitro assays of proliferative responses to nonspecific mitogens, alloantigen, and specific antigens.

TABLE 3. Effect of patient characteristics on the recovery of RRs to mitogens and MLC

Stimulus	Value for the indicated comparison[a]					
	Age, <5/≥5 vs <10/≥10 yr (K-W)		GvHD negative vs positive (M-W)		CMV positive vs negative (M-W)	
	RR (mo)	P	RR (mo)	P	RR (mo)	P
PHA, 5 μg/ml	12	0.033	1	0.024	6	0.001
	18	0.036	9	0.015	9	0.045
	24	0.025			18	0.017
ConA	18	0.004	9	0.028	6	0.014
			12	0.002	9	0.015
			18	0.010	18	0.040
			24	0.007	24	0.020
Alloantigen		0		0		0
PWM		0		0	6	0.005
					9	0.010
C. albicans		0		0		0

[a] K-W, Kruskal-Wallis; M-W, Mann-Whitney. The presence of GvHD and CMV positivity were associated with significant delay of immune function. Donor types, whether closely matched UD or partially matched FD, and cell dose did not affect the immune function recovery.

Immune recovery following BMT has been evaluated by several techniques, including determination of lymphocyte surface markers and functional analyses. T-lymphocyte function can be assayed in terms of the response to either nonspecific stimuli (e.g., PHA and ConA), specific antigenic stimulation (e.g., TT and herpesvirus antigens), or third-party alloantigen in MLC (20, 21, 44). In addition, other measures such as lymphokine production after mitogen stimulation, responsiveness to lymphokines, delayed-type hypersensitivity to recall antigens, and T-cell cytotoxicity to specific-antigen-bearing cells can be applied to assess T-cell function (37, 41). B-cell function after BMT can be evaluated by determination of serum immunoglobulin levels, proliferative response to B-cell mitogen (e.g., *Staphylococcus aureus* Cowan A), or specific-antibody production (e.g., to neoantigen such as keyhole limpet hemocyanin or phage φX174 (14, 21). Among these, mitogen blastogenic assays have been most commonly used to assess immune function (2, 15).

Proliferative responses to the T-cell mitogens in adults receiving non-T-cell-depleted transplants (2, 17, 39) have been extensively studied. Responses normalized approximately 6 months posttransplantation in patients without chronic GvHD and more slowly in those with this complication (1, 20). The lack of mitogen responsiveness may have reflected the deficiency of $CD4^+$ lymphocytes (22). While many NK cells appear after transplants (3, 15), these cells respond poorly to T-cell mitogens (14).

The effect of depletion of T lymphocytes from donor marrow on immune recovery is still controversial. In some studies, T-cell proliferative responses were delayed in recipients of T-cell-depleted grafts, normalizing as late as 18 months following transplantation (14, 29). Explanations included a lower rate of engraftment of myeloid and/or lymphoid cells as well as the removal of mature immunocompetent donor-derived cells from the graft (7). In contrast, another study found that hematopoietic and immunologic reconstitution in recipients of T-cell-depleted marrow was not different from that in T-cell-replete transplants (38). The proliferative response in MLC to third-party alloantigens, another measure of T-cell function, returned to normal within 6 months posttransplantation in T-cell-replete allografts (1, 20). However, MLC responses were depressed 12 months following transplantation in adults receiving T-cell-depleted (Campath-1) marrow (22). In our study, the proliferative responses to PHA, ConA, and PWM were impaired until 12 months following transplantation, while T-cell alloantigen responses normalized by 9 months.

We also assessed T-cell function by examining proliferative responses to specific antigens (streptokinase, TT, and *C. albicans* antigen). Since healthy individuals are genetically divided into responders or nonresponders to streptokinase (5, 12), we omitted this from further analysis. The responses to TT and *C. albicans* antigen were delayed and had not normalized by 36 months following transplantation. The timing of TT immunization is a critical factor for documenting a blastogenic response. In this study, some patients were not immunized until 18 to 24 months posttransplantation, which could explain our findings. Exposure to *C. albicans* most likely occurred before 18 months posttransplantation, and a lack of blastogenic responses to *Candida* antigen could reflect an immature immune system.

Other explanations could account for our observed delay in T-lymphocyte function. First, T-lymphocyte depletion of marrow removes a population of competent, fully differentiated cells that would function in a blastogenic response. Second, while immunosuppressive drugs control and minimize GvHD, they also are lymphocytotoxic and delay normal lymphocyte

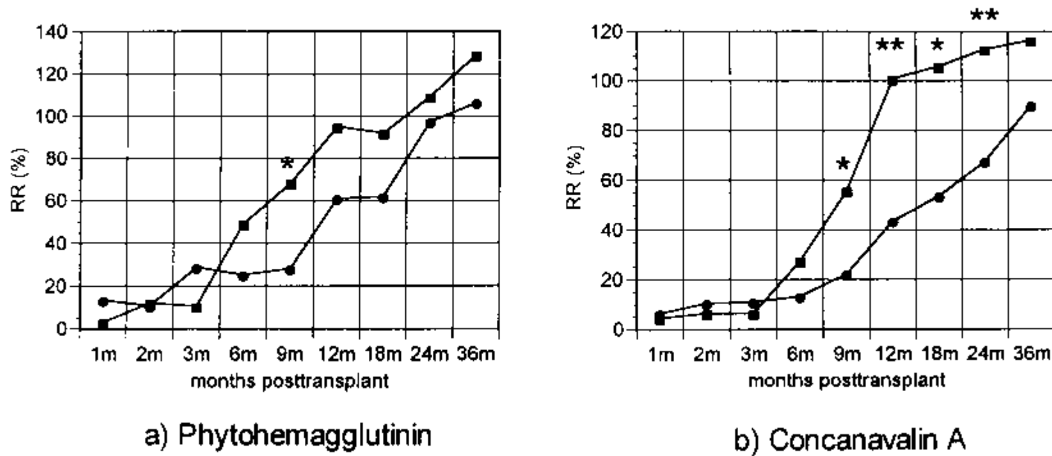

FIG. 3. RR to PHA (a) and ConA (b) according to GvHD status. ■, no GvHD; ●, GvHD of grade II or greater; ★, $P < 0.05$; ★★, $P < 0.01$. (GvHD status refers to all patients with acute GvHD stage II or greater and/or chronic GvHD.)

ontogeny (1, 24, 26). Furthermore, GvHD, which was present in a number of our patients, may select the lymphoid system as a target organ, further delaying immune recovery.

The second part of the study analyzed patient characteristics with respect to recovery of lymphocyte function. Younger patients tended to have better responses to PHA and ConA in the early posttransplantation period, but significant differences were not noted until 1 to 2 years after the transplants. Younger donors and younger recipients have previously been shown to be associated with higher mitogen responses as well as higher lymphocyte counts posttransplantation (15, 24).

Due to the limited number of patients in this study, patients with acute GvHD (grade II or greater) and/or chronic GvHD were grouped together. GvHD delayed normalization of proliferative responses to T-cell mitogens PHA and ConA until 24 months post-BMT. In contrast, GvHD did not affect the proliferative response to PWM or MLC responses to alloantigen. This is consistent with other studies where MLC responses to alloantigens were unaffected by GvHD (1, 19), although cytotoxic responses to alloantigen were delayed in patients with acute and chronic GvHD (19, 33).

Acute CMV infections are known to moderate the speed of immune recovery after BMT (23–25). CMV infections are associated with an increase in CD8 cells and a decrease in CD4 cells, resulting in an inverted CD4/CD8 ratio (15, 34, 43). Similar to the results in other reports we also found that CMV delayed lymphocyte mitogen responsiveness (18, 23, 34).

The degree of histocompatibility also delays immune reconstitution, resulting in higher infection rates in recipients of UD or HLA-nonidentical transplants than in recipients of matched sibling transplants (30, 40). In our study, we were unable to detect differences in immune function recovery between UD or partially matched FD, although we did not compare this for patients receiving transplants from matched siblings, due to differences in conditioning and GvHD prophylaxis and due to smaller patient numbers.

In conclusion, we examined recipients of T-cell-depleted BMTs for recovery of lymphocyte subpopulations and return of immune function. Factors affecting immune responses included the presence of GvHD and CMV and the total number of T lymphocytes. The CD4/CD8 ratio was quite variable and did not correlate with immune function, but the recovery of $CD3^+$ cells did correlate with the recovery of the T-cell mitogen responses. These studies may help guide clinicians in developing interventional strategies to prevent infections, knowing the degree of immune suppression present post-BMT, and in developing strategies to accelerate immune recovery in the post-BMT setting.

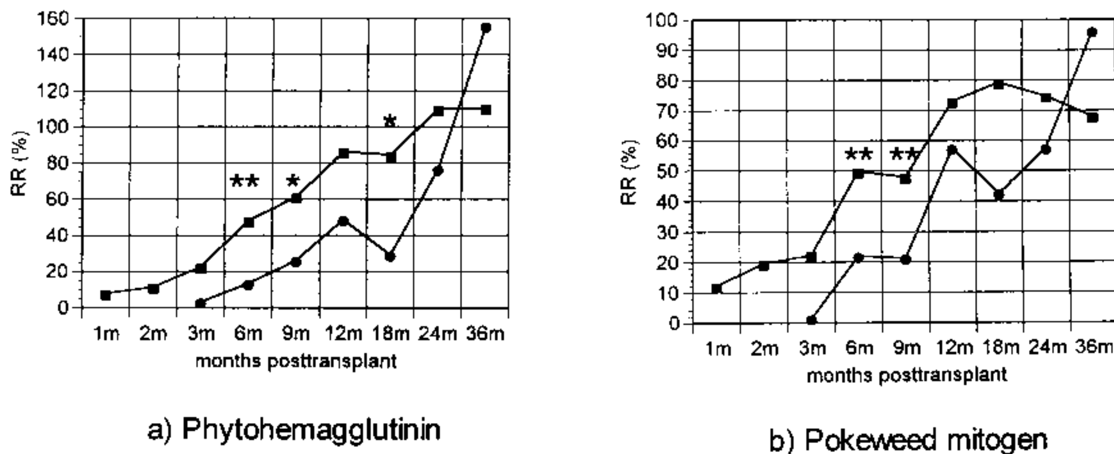

FIG. 4. RR to PHA (a) and PWM (b) according to CMV status. ■, CMV negative; ●, CMV positive; ★, $P < 0.05$; ★★, $P < 0.01$.

TABLE 4. Correlation of immunophenotypic data with immune function studies

Comparison	No.[a]	P for equal r[b]	Mean r[c]	Range[d]
Lymphocyte vs:				
PHA	22	0.396	0.542 (0.338–0.697)	
ConA	22	0.657	0.685 (0.524–0.799)	
Alloantigen	22	0.937	0.472 (0.250–0.647)	
PWM	22	0.177	0.518 (0.306–0.681)	
CD3 no. vs:				
PHA	22	0.117	0.582 (0.389–0.726)	
ConA	22	0.045		−0.190–0.998
Alloantigen	22	0.466	0.508 (0.294–0.674)	
PWM	22	0.084		−0.283–0.999
CD4 no. vs:				
PHA	20	0.737	0.460 (0.229–0.642)	
ConA	20	0.882	0.612 (0.419–0.753)	
Alloantigen	20	0.917	0.377 (0.129–0.581)	
PWM	20	0.758	0.405 (0.162–0.602)	
CD20 no. vs:				
PHA	20	0.000		−0.150–0.999
ConA	20	0.008		−0.150–0.998
Alloantigen	20	0.020		−0.386–0.998
PWM	20	0.022		−0.141–0.991
NK % vs:				
PHA	24	0.967	−0.332 (−0.527–−0.103)	
ConA	24	0.577	−0.495 (−0.655–−0.293)	
Alloantigen	24	0.962	−0.350 (−0.542–−0.123)	
PWM	24	0.972	−0.346 (−0.540–−0.118)	
CD4/CD8 ratio vs:				
PHA	25	0.016		−0.999–0.981
ConA	25	0.063		−0.998–0.967
Alloantigen	25	0.053		−0.992–0.987
PWM	25	0.041		−0.992–0.969

[a] Number of patients who had more than four matched pairs of data.
[b] P value of test of equal correlation among subjects ($P > 0.1$, acceptable equality).
[c] Mean correlation (95% confidence limits) by pooling across subjects (data shown only for those who had equal correlation).
[d] Range of correlation for those who had unacceptable equality.

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