The Regulation of Immunoglobulin Synthesis After HLA-Identical Bone Marrow Transplantation: VI. Differential Rates of Maturation of Distinct Functional Groups Within Lymphoid Subpopulations in Patients After Human Marrow Grafting

By Lawrence G. Lum, Margaret C. Seigneuret, Nancy Orcutt-Thordarson, Jan E. Noges, and Rainer Storb

RECIPIENTS of HLA-identical marrow grafts for hematologic malignancy or aplastic anemia have severely impaired immune functions in both the cellular and humoral arms of immunity. Although absolute numbers of T and B cells are normal within the first three months after marrow grafting, the patients can experience life-threatening infections with bacterial, viral, and opportunistic pathogens. The individuals who become long-term healthy survivors develop in vivo evidence for reconstruction of immunocompetent T and B cell compartments of their immune systems, whereas patients with chronic graft-versus-host disease (GVHD) continue to have impaired or altered in vivo and in vitro immune functions.

In this study, we used pokeweed mitogen (PWM), Epstein-Barr virus, herpes simplex type 1 virus, and tetanus toxoid systems as activators to elicit immunoglobulin production as measured by a plaque assay. Multiple defects in the various lymphoid subsets were found in both groups of patients. There was defective B cell function, lack of T cell or T4 cell subset helper activity, and increased suppressor T cell, T4 cell, or T8 cell suppressor activity after stimulation with the various activators. Inconsistent B, T, T4, and T8 cell functions in the marrow graft recipients provide evidence for (a) different functional groups of cells within each subset responsive to different polyclonal activators; (b) a spectrum of immune capabilities within each phenotypic lineage; (c) different patterns of immune reconstitution for each lymphocyte subset after marrow grafting; and (d) chronic GVHD altering recovery of in vitro functional responses to the different polyclonal activators.

MATERIALS AND METHODS

Patients

Twelve healthy long-term patients, one short-term patient with an unclassified pulmonary syndrome thought to be early chronic GVHD, and 12 long-term patients with chronic GVHD were studied after HLA-identical marrow grafting. The conditioning regimens for marrow transplantation have been described. Their unique patient numbers (UPN), pretransplant diagnoses, day of study after marrow grafting, circulating immunoglobulin (Ig) levels, specific antibody titers, history of herpes simplex infections, the proportion

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of T cells, and the percentage of T4 and T8 cells on purified E
rosette-positive cells, whenever available, are shown in Tables 1 and
2. Unless specifically noted, the marrow donors were immune to
tetanus toxoid and the transplant recipients were not re-immunized
with tetanus toxoid. Purified lymphoid subpopulations from their
marrow donors were used as controls whenever possible; otherwise,
lymphoid subpopulations from normal individuals were used as
controls. The lymphoid subsets from 12 of the 25 patients were
studied with the lymphoid subsets from their HLA-identical
donors. Syngeneic (identical twins) marrow transplants.

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Normal Ig ranges (mg/dL): IgA, 64–544; IgG, 408–1,788; IgM, 49–355; IgE, ≤200 IU/mL. Underlined Ig values are outside the normal range. See Table 1 for details on antibody titers to HSV and TT. UPN, unique patient number; D, recipient’s lymphocytes cocultured with unrelated cells; AA, aplastic anemia; ANL, acute nonlymphocytic leukemia; ALL, acute lymphoblastic leukemia; NT, not tested; ?, information not available.

*Syngeneic (identical twins) marrow transplant.
†Donor not immune to tetanus toxoid.

Table 1. Functional Diversity of T Cell Subsets

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†Donor not immune to tetanus toxoid.

Table 2. Patients With Chronic GVHD and Other Clinical Problems

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*This patient received a thymic transplant and developed signs of chronic GVHD before 100 days posttransplant.
†This juvenile CML patient developed an unclassified pulmonary syndrome (not idiopathic interstitial pneumonia, not due to opportunistic, mycobacterial, viral, fungal, or bacterial pathogens on extensively studied open-lung biopsy specimens).
using OKT8 and OKT4 monoclonal antibodies.\textsuperscript{32} Monocytes and other adherent accessory cell populations were not depleted from the non-T cell populations. The T cell populations contained >97\% E rosette-positive cells, <2\% surface Ig (slg)-positive cells and <2\% nonspecific esterase-positive cells.\textsuperscript{32} The non-T cell populations (designated B cells) contained no more than 3\% contaminating E rosette-positive cells and up to 30\% nonspecific esterase-positive cells.

\textbf{Lymphocyte Cultures for Ig Secretion}

Duplicate cultures were done in round-bottom Linbro (Hamden, Conn) microtiter plates at 37°C in a 5\% CO\textsubscript{2} humidified atmosphere.\textsuperscript{23,33} Each microwell contained from 2.5 \times 10\textsuperscript{4} to 1.5 \times 10\textsuperscript{5} T cells or T cell subsets. To facilitate direct comparisons between the various culture systems, all cultures contained 5 \times 10\textsuperscript{5} B cells. All cultures contained 250 \mu L of RPMI 1640 supplemented with penicillin-streptomycin, glutamine, and 10\% heat-inactivated fetal calf serum. Twelve hundred rad of irradiation to unfractionated T cells from a Cesium source before culture in the PWM system were used to optimize T helper activity. T cells or T cell subsets were not irradiated in the herpes simplex virus (HSV) or the tetanus toxoid (TT) system, since helper activity in both these systems is radiosensitive (see HSV- and TT-stimulated cultures).

\textbf{Hemolytic Plaque Assay Ig Secretion}

The number of Ig-secreting cells in each microculture was assessed using polyvalent developing antiserums specific for \(\alpha\), \(\gamma\), and \(\mu\) heavy chains in a plaque assay.\textsuperscript{24} The results are reported as plaque-forming cells (PFCs) per 10\textsuperscript{6} B cells cultured. All the data have been normalized to control T and B cell cultures (\(T_0 + B_N\)) in the various coculture experiments using the following expressions:

\[
\% \text{ control} = \frac{\text{experimental coculture}}{\text{control } T_0 + B_N} \times 100\%
\]

\[
\% \text{ suppression} = \left(1 - \frac{\text{control } T_0 + B_N + T_p}{\text{control } T_0 + B_N}\right) \times 100\%
\]

where \(T_p\) is the patient T cells or T cell subsets being tested for suppressor activity. All values presented in the figures have been corrected for unstimulated backgrounds. The control cultures for PWM, HSV, and TT are depicted in Figs 2 through 4. Each of the coculture combinations for the evaluation of B cell function, T cell helper activity, and T cell suppressor activity for each stimulant in the figures has been compared with the control culture containing appropriate numbers of normal T and B cells. B cell function or T cell helper activity <20\% of the control are considered defective. Moderate T cell suppression was defined as \(\geq 50\%\) suppression and potent suppression was defined as \(\geq 80\%\) suppression.

Each functional test performed has been labeled with patient-control pair numbers as assigned in Tables 1 and 2. Control responses and the actual PFC responses of the control T and B
for PWM, HSV, and TT are numbered in Figs 1 through 4.

Cultures with 5 x 10^6 B cells were added to each culture; 5 x 10^6 T cells were added to each culture; 5 x 10^6 T cells were added to each culture; 5 x 10^6 T cells were cultured with 5 x 10^6 B cells.

Cultures for PWM, HSV, and TT are numbered in Figs 1 through 4. Figs 5 through 11 have been normalized to the controls in Fig 2, 3, or 4 using the appropriate formulas.

**Pokeweed Mitogen-Stimulated Cultures**

PWM (GIBCO, Grand Island, NY) was used for these studies at a final concentration of 1:1,600 dilution of stock. In such cultures, 5 x 10^6 T cells or T cell subsets were added to 5 x 10^6 B cells to assess helper activity. Suppressor activity was assessed by adding 7.5 x 10^6 of the T cell population in question to 2.5 x 10^6 normal T cells and 5 x 10^6 normal B cells. The method for coculturing T cells, T cell subsets, and B cells has been described in detail. PWM cultures were harvested after six days of culture.

**Herpes Simplex Virus-Stimulated Cultures**

HSV type 1 antigen (HSV) used to stimulate Ig production was produced in monolayers of Vero cells (green monkey kidney) using the E115 strain of HSV as described. The system for the stimulation of Ig production using HSV antigen is as follows: (a) PBMCs from sero-positive individuals (enzyme-linked immunosorbent assay [ELISA] titers to HSV ≥1:16) can be stimulated with HSV to secrete IgA, IgG, and IgM, as detected by a plaque assay after six days; (b) the HSV system is T-dependent; and (c) T cell to non-T cell ratios of at least 2:1 were necessary for optimal Ig secretion (5 x 10^6 to 1 x 10^7 cells or T cell subsets were cocultured with 5 x 10^6 B cells). Our recent studies show that T4 cells from normal individuals are statistically better helper cells than are T8 cells (P = 0.002) and that T8 cells suppress Ig production better than T4 cells (P = 0.02, two-tailed Wilcoxon ranked sum).

**Fig 3.** HSV-stimulated autologous T cell and B cell coculture responses (PFCs per 10^6 B cells cultured) of normal controls (A); healthy survivors (B); patients with chronic GVHD (C). The control for each healthy survivor or patient with chronic GVHD has been identified by symbol (O or △) and specific number in the control panel. Medians are indicated -.

**Fig 4.** TT-stimulated autologous T cell and B cell coculture responses (PFCs per 10^6 B cells cultured) of normal controls (A); healthy survivors (B); patients with chronic GVHD (C). The control for each healthy survivor or patient with chronic GVHD has been identified by symbol (O or △) and specific number in the control panel. Medians are indicated -.

**Fig 5.** Summary of results of B cell function (5 x 10^6 patient B cells cultured with 5 x 10^6 normal T cells) from healthy survivors (A) and patients with chronic GVHD (B) after stimulation with EBV, PWM, HSV, and TT. Medians are indicated with —. The data for PWM, HSV, and TT are expressed as a percentage of the normal control cultures for PWM (Fig 2), HSV (Fig 3), and TT (Fig 4). The EBV data have been normalized to control B cell cultures containing 5 x 10^6 B cells (controls not shown).
Fig 6. Results for T cell and T cell subset helper function after each patient had been normalized to the appropriate control culture in Fig 2 for PWM. The data are expressed as a percentage of the numbered control T + B cell culture. Medians are indicated with →. The control cultures contained 5 x 10⁶ T and 5 x 10⁶ B cells. (A) Healthy survivors; (B) patients with chronic GVHD.

Fig 7. Results for T cell and T cell subset helper function after each patient had been normalized to the appropriate control culture in Fig 3 for HSV. The data are expressed as a percentage of the numbered control T + B cell culture. Medians are indicated with →. The control cultures contained 5 x 10⁶ T and 5 x 10⁶ B cells. (A) Healthy survivors; (B) patients with chronic GVHD.

Fig 8. Results for T cell and T cell subset helper function after each patient had been normalized to the appropriate control culture in Fig 4 for TT. The data are expressed as a percentage of the numbered control T + B cell culture. Medians are indicated with →. The control cultures contained 1.5 x 10⁶ T and 1.5 x 10⁶ B cells. (A) Healthy survivors; (B) patients with chronic GVHD.

Fig 9. Summary of ability of T cell and T cell subsets to exert suppressor activity when mixed with control T and B cells after stimulation with PWM. The numbered controls for these data are in the left panel of Fig 2. Medians are indicated with →. The control cultures contained 2.5 x 10⁶ T and 5 x 10⁶ B cells. (A) Healthy survivors; (B) patients with chronic GVHD.
cells to 5 x 10^5 B cells. Control cultures contained 1 x 10^5 or 1.5 x 10^5 T cells and T cell helper activity were compared with the control PFCs obtained using these cell numbers. Suppression in the HSV system was assessed by adding 5 x 10^5 patient T cells or T cell subsets to 5 x 10^4 normal T cells and 5 x 10^5 normal B cells. Tables 1 and 2 show HSV titers, patient numbers, and whether the patient's cells were cocultured with donor or unrelated control cells.

**Tetanus Toxoid-Stimulated Cultures**

We recently described a TT-stimulated culture system to stimulate PBMCs from immune but not boosted normal subjects to produce IgG. The system can be summarized as follows: (a) PBMCs can be induced by TT to produce IgA, IgG, and IgM, as measured by a plaque assay after eight to nine days of culture; (b) unstimulated PBMCs or B cells do not produce significant numbers of PFCs; and (c) the system T-dependent and optimal Ig secretion occurs at T to B ratios of at least 3:1. The TT (purchased from Commonwealth of Massachusetts, Department of Public Health, Division of Biologic Laboratories, Lot #LP445PR) was used at a final concentration of 1 Lf/mL. Optimal PFC formation was obtained by adding 1.5 x 10^5 T cells to 5 x 10^5 B cells. Control cultures contained 1 x 10^5 or 1.5 x 10^5 T cells and 5 x 10^5 B cells. When cell numbers permitted, both T cell concentrations were cultured with control B cells. Assays for B cell function and T cell helper activity were compared with the designated control value. Suppressor activity was assessed by adding 5 x 10^5 T cells to 1 x 10^5 normal T cells and 5 x 10^5 B cells and by normalizing the PFCs obtained to the designated control value.

T4 cells are better helper cells than are T8 cells (P < .01), and T8 cells are better suppressor cells than are T4 cells (P < .01, Wilcoxon matched-pairs signed-ranks test) in this system. T cell or T4 cell helper activity was radiosensitive to 1,200 rad. The T4 helper activity induced by TT was significantly less than the T4 helper activity induced by PWM (P < .01, Wilcoxon matched-pairs signed-ranks test).

Only the coculture combinations in which the control cultures responded to TT were evaluated and presented. Tables 1 and 2 show the TT titers, history of booster immunizations after transplant, identifying patient numbers, and their respective donor or unrelated control.

**Epstein-Barr Virus-Stimulated Ig Secretion**

The production of Epstein-Barr virus (EBV) and the EBV stimulation of purified non-T cells for in vitro polyclonal Ig production has been outlined. Fifty microliters of EBV containing supernatants was added to each microwell containing 5 x 10^4 non-T cells (final volume - 250 μL). T cells were not added to EBV-driven cultures. The cultures were assessed for PFCs after eight days.

**Effects of Allogeneic Cultures**

We have previously shown that allogeneic mixtures for B cell function or T helper activity resulted in 12% enhancement and 45% enhancement, respectively, over the autologous cocultures in the PWM system. Allogeneic cocultures in both the HSV and TT systems have led to similar results (data not shown). There is no evidence for allogeneic restriction in any of the systems used. Therefore, B cell defects, lack of T helper activity, and T cell suppressor activity occur despite allogeneic enhancing effects.

**Statistics**

Statistical comparisons were done using two-tailed Wilcoxon rank sum analyses or two-tailed Wilcoxon matched-pairs signed-ranks test as indicated. The matched-pairs signed-ranks test was applied when paired control data were available.
RESULTS

Heterogeneous Functional Responses to Different Polyclonal Activators by the Same Lymphocyte Subset

Figure 1 shows how the various lymphoid subpopulations from one patient were cocultured with donor lymphoid subsets in the presence of PWM, HSV, and TT. The results show that a specific function elicited from a certain lymphoid subset by one activator cannot always be elicited by a different activator. In other words, different or even opposing immunoregulatory functions may be expressed by the same subset after stimulation with different stimuli. The remarkable findings for this transplant recipient are (a) T4 cells help after PWM stimulation but do not help after HSV or TT stimulation, and (b) T4 cells enhance in the suppressor assay after PWM stimulation while acting as potent suppressor cells after TT stimulation.

The complexity of the functional data provided the rationale for presenting the same data in both tabular (Tables 3 and 4) and graphic (Figs 2 through 11) forms for the sake of readability and comprehension. The functional profiles in Tables 3 and 4 are derived from the normalized data in Figs 2 through 11. Tables 3 and 4 show the individual functional profiles of 12 long-term healthy marrow graft recipients and 13 marrow graft recipients with chronic GVHD. Figs 2 through 11 display PFC data or normalized PFC data for each marrow graft recipient. Generally, the functional responses of a given subset from a particular marrow graft recipient after stimulation with one activator was similar to the functional responses elicited by the other activators. However, as indicated in these tables and figures, not one of the marrow graft recipients has a lymphocyte subset that responded in the same functional manner to stimulation with these different polyclonal activators. The functional profiles for the 25 patients show how each subset responded to the various activators. These data show the functional heterogeneity within each lymphocyte subset.

Autologous PFC Responses of T and B Cells From Control Individuals and Marrow Graft Recipients

Figures 2 through 4 summarize the autologous PFC responses of cells from 12 long-term healthy survivors, 13 patients with chronic GVHD, and their controls after PWM, HSV, and TT stimulation. The median control PFC responses, as indicated in Figs 2, 3, and 4, were 35,000 PFCs per $10^6$ B cells cultured, 6,500 PFCs per $10^6$ T cells cultured, and 10,200 PFCs per $10^6$ B cells cultured for PWM, HSV, and TT, respectively. Statistical comparisons between the normal control T and B cell PFC responses and the autologous T and B cell PFC responses of the recipients showed both patient groups to be significantly lower in their responses in the PWM and TT systems ($P < .01$, matched-pairs signed-ranks). In the HSV system, the healthy survivors were not different from normal and those with chronic GVHD were significantly lower than normal individuals ($P < .01$, matched-pairs signed-ranks test).

Table 5 shows the results of statistical comparisons between the healthy survivors and recipients with chronic GVHD. Although the autologous responses of T and B cells from the patients with chronic GVHD appear to be more impaired than their healthy counterparts in Fig 2, there were no statistical differences between the autologous T and B cell responses of the healthy survivors and the patients with chronic GVHD after PWM, HSV, and TT stimulation.

### Table 3. Functional Profiles of Long-term Healthy Survivors

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+ /total* 3/6 6/12 8/12 5/12 8/12 8/12 5/12 9/9 8/8 4/8 1/9 2/8 0/8 5/11 2/11 2/11 2/10 0/8 3/8 8/10 5/6 6/8

% 50 50 66 42 66 66 42 100 100 50 11 25 0 45 18 18 20 0 38 80 83 75

Normal B cell function is defined as PFC > 20% control. Normal T cell helper activity is defined as PFC ≥ 20% control. Suppressor T cell activity is defined as ≥50% suppression of the control culture. Blank spaces indicate that the test was not performed. +, function present; -, function absent.

*Fraction of functions present over individuals tested.
Heterogeneity of B Cell Function

Figure 5 shows the results for patient B cells cultured in the presence of optimal numbers (as indicated in Materials and Methods) of normal T cells after stimulation with PWM, HSV, or TT. The B cell PFC responses to EBV shown in Fig 5 were assessed in the absence of T cells. Control EBV-driven B cell PFC responses were consistently greater than 1,000 PFCs per 10⁶ B cells cultured after eight days of culture (data not shown). Tables 3 and 4 show the functional profiles for each patient's B cell PFC responses. Table 5 shows the results of statistical comparisons between the healthy long-term survivors and the patients with chronic GVHD for B cell function. The B cell function of patients with chronic GVHD was significantly impaired when compared with the B cell function of healthy long-term survivors in the PWM, HSV, and TT systems (Table 5). B cell function of the healthy survivors in the HSV system and their paired normal controls were not statistically different.

Heterogeneous T Cell and T Cell Subset Helper Activity

T cells or T cell subsets from healthy long-term survivors and patients with chronic GVHD were assessed for helper activity after stimulation with PWM, HSV, and TT (Figs 6 through 8). Tables 3 and 4 show T cell and T cell subset helper activity responses for each marrow graft recipient after stimulation with the various stimulators. Defective T cell helper activity was more frequent in the patients with chronic GVHD. Defects in T cell helper activity occurred most often in the TT system. T cells from chronic GVHD patients were more impaired in helper activity than the T cells from the healthy long-term survivors after stimulation with HSV or TT (P = .002 and P = .05, respectively). The T4 subsets from the healthy long-term individuals provided helper activity to normal B cells in all nine healthy survivors studied after PWM stimulation and all eight studied after HSV stimulation. However, four of eight healthy survivors had impaired T4 helper activity after activation with TT. In contrast, four of five patients with chronic GVHD had impaired T4 helper activity after TT stimulation.

Heterogeneous T Cell and T Cell Subset Suppressor Activity

Figures 9 through 11 summarize the normalized data for suppressor activity exhibited by T cells from
healthy survivors and patients with chronic GVHD after activation with PWM, HSV, and TT. Tables 3 and 4 summarize the functional profiles of the patients whose T cells or T cell subsets were assessed for suppressor activity. In all the systems tested, T cells and T cell subsets from the patients with chronic GVHD suppressed Ig production more frequently than did the T cells and T cell subsets from the healthy marrow graft recipients (Tables 3 and 4). T, T4, and T8 cells from the patients with chronic GVHD were more suppressive than the respective subpopulations from the healthy long-term survivors after HSV stimulation (Table 5). After TT stimulation, T cells and T8 cells from patients with chronic GVHD were significantly better suppressor cells than the same populations from the healthy long-term survivors (P = .05 and .04, respectively). There were no differences in the ability of the various T cell subsets to suppress Ig synthesis in the PWM system.

**Correlations Between Serum Titers and In Vitro Immune Responses to the Various Polyclonal Activators**

There were no correlations between the presence of serum IgG anti-TT or anti-HSV antibody titers and the presence of in vitro immune responses such as autologous T and B cell PFC responses, B cell function, T cell or T cell subset helper activity, and T cell or T cell subset suppressor activity after stimulation with TT or HSV, respectively. In addition, there were no correlations between PWM-stimulated functions and serum Ig levels. Similarly, the presence or absence of specific anti-TT or anti-HSV titers did not correlate with PWM-stimulated T and B cells functions.

**DISCUSSION**

Many investigations have documented the immunodeficiency after human bone marrow transplantation using in vivo and in vitro immune function tests. However, none of the previous studies has taken our present approach. By using different polyclonal activators to induce Ig production, this investigation shows that (a) there are different functionally reactive groups in any particular individual’s lymphocyte subset; (b) there is functional diversity in each phenotype; (c) the different lymphocyte subsets gain function according to their own intrinsic schedules; and (d) chronic GVHD affects the development of lymphocyte subset functions. This investigation shows that a spectrum of functional responses to different signals within each phenotypic lineage can be observed after bone marrow transplantation. These findings may reflect developmental stages within the lymphocyte subsets.

PWM, HSV, TT, and EBV elicited different functional responses from each of the various lymphocyte subsets and identified different functional groups within any given recipient’s particular lymphocyte subset. In normal individuals, T4 cells are consistent helper cells and T8 cells are consistent suppressor cells in the PWM, HSV, and TT systems. In marrow graft recipients, the results for any particular recipient’s cells show that a given lymphocyte subset’s response varies as a function of the agent used to induce Ig production. Tables 3 and 4 illustrate that a given subset for a particular recipient may respond to one activator of Ig production and not to another.

The differing patterns of immunoreactivity of B cells to the various activators exemplify the point discussed earlier. B cells from healthy survivor No. 12 did not respond to HSV and TT, but responded well to PWM. On the other hand, B cells from healthy survivor No. 5 responded well to HSV but not at all to EBV, PWM, and TT. There were also B cell populations that responded to all or none of the activators. Such data suggest that mitogen-specific precursor cells (clonal groups) must be present and susceptible to activation by the appropriate signal in order to produce Ig. Similar findings are seen in the assays for helper and suppressor function using T, T4, or T8 cells from transplant recipients. Normal B, T, T4, and T8 cells do not have such large variations in their functions in the PWM, HSV, and TT systems. Such data provide evidence for the existence of different groups of clones within the different lymphocyte subsets. Each group is capable of responding uniquely to each activator. Each activator may stimulate different clonal groups of cells but with different degrees of potency or the different activators may stimulate responses from different but not mutually exclusive reactive groups within the large array of cells that can be activated. Heterogeneous PFC reactivity and the variation in the magnitude of PFC responses from a specific marrow recipient’s lymphocyte subset in response to different activators support the latter explanation.

A variety of functions or lack of function can be exhibited by the same T cell phenotype purified from different bone marrow recipients. T4 cells from different recipients, for example, after stimulation with PWM could exhibit helper activity, lack of helper activity, or potent suppressor activity as a function of the recipient from whom the cells were obtained. Generally, T8 cells suppressed Ig production in the PWM, HSV, and TT systems; in a number of cases, however, functional diversity was observed in T8 cells. T8 cells from healthy survivor No. 8 exhibited helper activity in the PWM and HSV systems. T8 cells from long-term healthy survivors No. 2, 3, 8, and 12 and
chronic GVHD patient No. 3 did not suppress Ig production in the PWM, HSV, and TT systems. There was functional diversity within each of the recipient's T cell helper and suppressor-cytotoxic phenotypes in all of the Ig production systems tested. The results of this investigation strengthen the hypothesis that such heterogenous populations do exist within well-defined subsets as exemplified by the enrichment of different functions in any particular subset purified from different recipients.

The different proportions of intact B cell, T cell, T4 cell, and T8 cell functions after stimulation with the different activators support the contention that different clonal groups reactive to a specific stimulus may gain responsiveness at unique times after marrow grafting. In the analysis of B cell function and T or T4 cell helper function, the proportion of positive responses to HSV stimulation was higher than the proportion of positive responses to TT stimulation except for B cell responses of the recipients with chronic GVHD. The results indicate that the ability of the lymphocyte subsets to respond to HSV stimulation may have developed earlier in time than the ability of the same subsets to respond to TT stimulation. B cell defects were highest after TT stimulation in both patient groups. Such differential responsiveness to different probes could not have been investigated using PWM alone.

The differences in function were most dramatic in the helper activity expressed by the T4 cells from both the long-term healthy survivors and the recipients with chronic GVHD. In the long-term healthy survivors, the number of recipients who had T4 helper responses in the normal range induced by HSV were double that induced by TT. Similarly, T4 cells from the recipients with chronic GVHD had almost three times more helper activity in the HSV system than in the TT system. Such differences in T4 helper activity between the HSV system and the TT system suggest that helper responses recovered sooner to HSV stimulation than to TT stimulation irregardless of the presence of chronic GVHD. Reexposure to HSV antigen (active infection) postgrafting may be responsible for higher HSV-stimulated responses in vitro. Even in the presence of chronic GVHD, the capacity of a particular group of cells within a particular lymphocyte subset to respond or not respond to stimulation with a particular mitogen was evident.

The increased frequency of defective or altered T, T subset, and B cell functions in transplant recipients affected with chronic GVHD shows the negative influence of chronic GVHD on the normal development of lymphocyte subsets. Healthy survivors had fewer defects in their subsets than did the patients with chronic GVHD. Earlier studies using PWM showed increased frequency of B cell defects, T helper cell defects, and T4 helper cell defects and increased suppressor activity in T cells, T4 cells, and T8 cells in marrow graft recipients with chronic GVHD. This study shows that similar functional defects occur in the HSV, TT, and EBV systems. However, the critical new finding is that an abnormality or alteration of function in a particular lymphocyte subset in one particular system cannot be generalized to another system.

Our current findings demonstrate that immune reconstitution occurs in a variable fashion associated with extremely heterogeneous B and T cell subset functions. First, there are functionally distinct cell groups within each lymphocyte subset. Second, there is functional heterogeneity within each T lymphocyte phenotype. Third, each functional cell group within a phenotype may gain its ability to respond to a particular stimulus at a unique time after marrow grafting. Fourth, chronic GVHD has a strong negative effect on the redevelopment of normal functioning lymphocyte subsets and the various cell groups that reside within each lymphocyte subset. Fifth, the response or nonresponse of each subset may depend on in vivo reexposure to the specific stimulus (eg, HSV or TT).

The varied and heterogenous functions within each subset may represent the activity of maturational stages that occur during immune reconstitution after bone marrow transplantation. The process of immune reconstitution may permit the expression of maturational stages that have different functions within a phenotypic lineage; these functions exist only transiently in the normal course of immune ontogeny. Alternatively, different or diverse functional responses to a specific polyclonal activator by a particular T cell phenotype may represent different activational states within a specific subset. In the former case, suppressor activity by T cell subsets may be a primitive function present early after grafting, and helper activity may be a more mature, differentiated function that appears later in all systems used in this study. Chronic GVHD may cause arrests or delays in a particular subset's maturational pathway, thereby leading to in vivo enrichment for a particular function within a subset. This study underscores the need for caution in the interpretation of phenotyping data in patients with altered immune states in the absence of functional assays.

This investigation shows that B cell functions and T cell regulatory functions stimulated with PWM, HSV, or TT do not correlate with Ig levels, IgG anti-HSV or IgG anti-TT on a patient-per-patient basis. Such discrepancies between in vivo and in vitro functions point out the need for new assays that relate in vitro
antigen-induced antibody synthesis to in vivo antibody synthesis for analyzing these complex patients.

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REFERENCES

31. Lum LG, Muchmore AV, Keren D, Decker J, Koski I.


34. Lum LG, Orcutt-Thordarson N, Seigneuret MC: Regulatory roles of human OKT4/OKT8 subsets in polyclonal immunoglobulin production induced by herpes simplex type 1 virus. Immunobiology (in press)


42. Azogui O, Gluckman E, Frodelizi D: Inhibition of IL 2 production after human allogeneic bone marrow transplantation. J Immunol 131:1205, 1983


The regulation of immunoglobulin synthesis after HLA-identical bone marrow transplantation: VI. Differential rates of maturation of distinct functional groups within lymphoid subpopulations in patients after human marrow grafting

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