In Vitro Regulation of Immunoglobulin Synthesis after Human Marrow Transplantation. II. Deficient T and Non-T Lymphocyte Function within 3–4 Months of Allogeneic, Syngeneic, or Autologous Marrow Grafting for Hematologic Malignancy

By Robert P. Witherspoon, Lawrence G. Lum, Rainer Storb, and E. Donnall Thomas

Immunoglobulin secretion was studied in 37 patients between 19 and 106 days after allogeneic HLA-identical (30 patients), allogeneic one HLA-haplotype-identical (three patients), syngeneic (three patients), or autologous (one patient) marrow grafting. E rosette-positive (T) and E rosette-negative (non-T) peripheral blood mononuclear cells were cocultured with pokeweed mitogen for 6 days. Polyvalent immunoglobulin secretion was determined by counting plaque forming cells in a reverse hemolytic plaque assay. The number of antibody secreting cells in cocultures of autologous T and non-T lymphocytes was low in 40 of 44 tests conducted on samples from the 37 patients.

Morbidity and Mortality during the first 3–4 mo after marrow grafting for acute leukemia result largely from opportunistic infection with viruses or Pneumocystis carinii. Approximately 25% of marrow graft recipients die of interstitial pneumonia associated with these agents. The development of infection is presumably due in part to impaired humoral and cellular immune responses in vivo observed in these patients during the first 3–4 mo posttransplant.

Cocultures of patients' lymphocytes with staphylococcal bacteria within 4 mo after grafting failed to yield antibody producing cells. The present study was designed to clarify the cellular mechanisms responsible for deficient antibody production in the first 3–4 mo after grafting. T and non-T lymphocyte subpopulations from patients grafted for leukemia were used in coculture with the T-dependent polyclonal activator pokeweed mitogen.

Materials and Methods

Patients and Marrow Transplantation

Thirty-seven patients were transplanted for acute leukemia as previously described.11 Three received marrow from syngeneic donors, 30 from allogeneic HLA-identical donors, three from HLA-haplodendent donors (all three were genotypeiy identical to their recipient for one chromosome; on the other chromosome two were identical for B and D but mismatched for A, and the remaining donor was mismatched for A, B, and D), and one was the recipient of autologous marrow stored at –70°C. Briefly, the patients were treated with 120 mg/kg of cyclophosphamide followed by 1000–1200 rad total body irradiation. Allogeneic recipients were treated with Methotrexate for 3 mo to prevent graft versus host disease (GVHD). The one phenotypically haploidentical patient received 880 mg Cyclosporin A from days 0–12 and 440 mg from days 16–21 postgrafting, and did not receive methotrexate. Two of the three syngeneic and one autologous transplant recipients did not receive Methotrexate. All patients with infection were treated with appropriate antibiotics and studied when their clinical condition was stable. Acute GVHD was diagnosed by skin, liver and gastrointestinal abnormalities as previously described13 and seven of the allogeneic recipients received prednisone as treatment for acute GVHD.

Cultures

Mononuclear cells were obtained from heparinized peripheral blood by Ficoll-Hypaque (F/H) density gradient centrifugation at 1800 × g for 7 min. The entire mononuclear cell suspension was mixed with 2-aminoethylisothiouronium bromide modified sheep red blood cells, placed over a cold F/H density gradient, and subjected to centrifugation at 400 × g for 20 min and 1800 × g for 7 min. The E-rosette-positive pellet (T cells) was freed of sheep red blood cells with ammonium chloride tris hemolytic buffer. The interface (non-T cells) and the T cells were washed four times and resuspended at a concentration of 1 × 10⁶ lymphocytes per milliliter. All cultures were done in duplicate in round-bottom microtiter plates (Flow Laboratories, Hamden, Conn.) for 6 days with or without pokeweed mitogen (PWM) (GIBCO, Grand Island, N.Y.) in RPMI 1640 (GIBCO) supplemented with penicillin-streptomycin, 4 mM glutamine, and fetal calf serum (Reheis, Phoenix, AZ). A typical experiment shown in Fig. 1 consisted of coculturing 5 × 10⁴ and 5 × 10⁵ non-T lymphocytes of patients and normal individuals. When cell recovery from patients was poor, only unfractionated mononuclear cells (5 × 10⁴) were used with purified T or non-T populations from the normal individual. To assess suppressor activity, patient T cells (5 × 10⁴) were cocultured with normal T (5 × 10⁴) and non-T
yield of patient cells was too poor to do separations. Cultivated mononuclear cells were substituted for T or B when the cells. T = normal T cells. BN = normal non-T cells. B, = patient T cells irradiated with 1200 rad. B, = patient non-T cells. Unfractionated mononuclear cells were substituted for T, or B, when the yield of patient cells was too poor to do separations.

lymphocytes \(5 \times 10^8\). To assess immunoglobulin (Ig) secretion in allogeneic cocultures of T and non-T cells from two unrelated individuals, cocultures identical to those specified in Fig. 1 were done. Normal or patient T or non-T lymphocytes cultured separately with PWM did not secrete Ig.

**Plaque Assay**

The cells in each culture well were washed and the entire contents plated in the plaque assay as previously described\(^{15,16}\) using a rabbit polyvalent antibody (identifying \(\alpha, \mu\) and \(\gamma\) determinants) raised locally against human Cohn fraction II. The number of plaques in each culture were enumerated and expressed as though \(5 \times 10^7\) non-T lymphocytes were cultured. To facilitate comparisons between individual patients, non-T and T helper cell function was expressed as a per cent of the number of plaques counted for normal individual's lymphocytes studied in the experiment that day according to the following formulas:

**Formula 1.** Per cent B cell function:

\[
\frac{B_T + T_P}{B_N + T_N} \times 100\%
\]

**Formula 2.** Per cent T helper function:

\[
\frac{T_P + B_P}{T_N + B_N} \times 100\%
\]

Suppressor cell function was expressed according to this formula:

**Formula 3.** Percent T suppressor function:

\[
\left(1 - \frac{T_P + T_N + B_P}{T_N + B_N}\right) \times 100\%
\]

B = non-T cell fraction.
T = T cell fraction.
\(T^*\) = T cells irradiated with 1200 rad.

Subscript P = patient cells.
Subscript N = normal cells from unrelated individuals.

If unfractionated mononuclear cells rather than purified subpopulations from the patient were used, the number of antibody producing cells in the mononuclear fraction alone was subtracted from the number in the cocultures with normal non-T cells to calculate helper and suppressor activity.

**Serum Immunoglobulin Levels**

These studies were kindly performed using standard methods in the clinical laboratory of the Department of Laboratory Medicine, University of Washington.

**Informed Consent**

All studies described herein were performed under protocols approved for that purpose by the Human Subjects Review Committees of the University of Washington and the Fred Hutchinson Cancer Research Center.

**RESULTS**

**Autologous T and Non-T Cell Cocultures**

The first two columns of Fig. 2 show the number of plaque forming cells from autologous T and non-T lymphocytes of 28 normal individuals and the 42 cultures in 37 patients. The remaining four columns show subgroups (those from allogeneic recipients without or with acute GVHD, infection without GVHD, or from syngeneic or autologous graft recipients) of data from the second column. Antibody production of autologous T and non-T cells from patients of each group was similar. Lymphocytes from one syngeneic (the one receiving Methotrexate) and three HLA-identical allogeneic recipients were in the range of the normal
individuals, but lymphocytes from the remainder of patients made few or no plaques.

**Non-T Cell Function**

Fig. 3 shows results for non-T cell function for each of 40 cultures normalized to percent of the control in each experiment according to formula 1. One syngeneic (receiving Methotrexate) and one HLA-identical allogeneic recipient demonstrated non-T cell function within the 5th to 95th percentile of the normal distribution, and the remaining patients were below the 5th percentile. The function of lymphocytes from patients who did or did not have acute GVHD or infection or who were recipients of syngeneic or autologous marrow grafts was similar.

**T Helper Function**

Fig. 4 shows T helper function for each culture normalized according to formula 2. Fifteen of the 37 cultures in 31 patients had T helper function within the 5th–95th percentile range of normal controls, and the remaining cultures were below the 5th percentile. Lymphocytes from patients with or without acute GVHD or infection or from recipients of syngeneic or autologous marrow grafts were similar. Lymphocytes from one recipient of partially histoincompatible mar-

![Fig. 3](image)

**Fig. 3.** Percent normal non-T cell function in 40 cultures from 37 patients expressed according to formula 1:

\[
\frac{B_T + T_{N}^*}{B_n + T_{N}} \times 100\% 
\]

The median and 5th to 95th percentile intervals of the percent responses when lymphocytes of two unrelated normal individuals were co-cultured (N = 18 cultures) are shown with dotted lines. Data from all patients are shown in the left hand column (0). Those from allogeneic recipients with or without acute GVHD or infection and from syngeneic or autologous recipients are shown separately to the right. See legend for Fig. 2.

![Fig. 4](image)

**Fig. 4.** Percent normal helper T-cell function in 37 cultures from 31 patients expressed according to formula 2:

\[
\frac{T_h + B_{N}}{T_N + B_{N}} \times 100\%
\]

The median and 5th to 95th percentile intervals of percent responses when two unrelated normal individuals were co-cultured is shown with dotted lines (N = 20 cultures). See legend for Figure 2.

row (genotypically mismatched at HLA-A) had normal T helper function. The other two showed function at and below the 5th percentile level of normal function.

**T Cell Suppressor Activity**

Fig. 5 shows T cell suppressor activity for each culture normalized according to formula 3. When lymphocytes of normal individuals were cocultured, the greatest suppression was 47% and enhancement was 600%. T cells of 25 of 41 cultures suppressed normal T and B lymphocyte function greater than 50%, and T cells from the remaining cultures did not achieve 50% suppression. Twenty-three of these tests demonstrated greater than 80% suppression. One syngeneic recipient not receiving Methotrexate showed enhancement. Lymphocytes from one of the three recipients of partially histoincompatible marrow demonstrated suppression. Suppression could be reversed in 17 of the 25 cultures by in vitro irradiation of T cells with 1200 rad. The results of those 17 patients whose suppressor function reversed ranged from 19% suppression to 500% enhancement (median 41% enhancement) after irradiation. Those eight patients whose suppressor function did not reverse ranged from 51% to 100% suppression (median 79% suppression) after irradiation.

Table 1 summarizes the types of in vitro lymphocyte abnormalities noted in the various clinical conditions in the first 100 days after marrow grafting.
LYMPHOCYTE FUNCTION AFTER MARROW GRAFTING

The remaining 16 cultures were done with the purified T fraction. The remaining 25 cultures were performed with purified non-T fraction.

Six of the 25 tests showing T suppression were actually done with unfractionated mononuclear cells. The remaining cultures were done with the purified T cell fraction.

DISCUSSION

Opportunistic infection in the first 3–4 mo after marrow grafting is more common in allogeneic than in syngeneic recipients. Among allogeneic recipients, those with acute GVHD have infections more commonly than those without acute GVHD. Yet to our surprise, in vivo cellular and humoral immunologic reactivity during the first 3–4 months after grafting in syngeneic recipients was no better than that in allogeneic recipients, and allogeneic recipients without acute GVHD were no better than those with acute GVHD.

The present study was undertaken to explore in vitro immunologic reactivity of lymphocyte subpopulations as a possible explanation for the different frequencies of infection in spite of comparable in vivo immunologic reactivity among allogeneic recipients with and without GVHD and syngeneic recipients. The in vitro results were consistent with the in vivo findings and showed that the defects in syngeneic or allogeneic recipients were similar and due to a spectrum of abnormalities. First, the non–T cell fraction containing B cells failed to produce Ig. Secondly, T helper function was absent or greatly reduced in many of the patients. Thirdly, excessive T suppressor cell activity was noted.

All the patients lacked functioning antibody producing cells in the non–T fraction. One cannot be certain that the deficiency was in B cells alone, however. The role of the monocyte in the non-T cell fraction is unclear because experiments involving removal and readdition of monocytes were not attempted. Monocytes represented no more than 20% of the total non–T cells in representative observations.

Table 1. Summary of Deficiencies in Marrow Transplant Recipients

<table>
<thead>
<tr>
<th>Recipients Studied</th>
<th>Non-T failure</th>
<th>T-helper failure</th>
<th>T suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allogeneic—Non-GVHD</td>
<td>10/10⁶</td>
<td>7/10</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>Allogeneic—GVHD</td>
<td>17/18</td>
<td>9/17</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>Allogeneic—Infection</td>
<td>8/8</td>
<td>3/6</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>Syngeneic and autologous</td>
<td>3/4</td>
<td>4/4</td>
<td>&gt;50%</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td>39/40</td>
<td>23/37*</td>
<td>25/41¹</td>
</tr>
</tbody>
</table>

*Failure defined as <5th percentile of distribution of percent normal function outlined in text.
²Ratio of the number of tests showing defects to the number of tests done.
³Patients with infection had not experienced GVHD at the time of testing.
⁴Thirteen of the 38 tests showing non-T failure were actually done with unfractionated mononuclear cells which exhibited either no or radiosensitive suppression. The remaining 25 cultures were performed with purified non-T fraction.
⁵Seven of the 23 tests showing T helper failure were actually done with mononuclear cells which exhibited either no or radiosensitive suppression. The remaining 16 cultures were done with the purified T fraction.
⁶Six of the 25 tests showing T suppression were actually done with unfractionated mononuclear cells. The remaining cultures were done with the purified T cell fraction.
decreased proportions of circulating T helper cells identified with the OKT4 phenotype. The lack of helper T cell activity was noted in T lymphocytes not having suppressor activity as well as in radiosensitive suppressor T cell populations. The remaining six patients had radioresistant (1200 rad) suppressor cells which made the assessment of helper activity impossible. Radiosensitive helper activity was detected in one patient (UPN 1166). Other patients without suppression did not have helper activity that was sensitive to irradiation.

Half the patients had suppressor T cells, a finding consistent with the elevated proportions of circulating T cells bearing the OKT8 suppressor phenotype. In one third of the patients the suppressor T cells were radiosensitive. The altered proportion of OKT4+/OKT8+ T cell subpopulations, the presence of suppressor activity and lack of helper activity in the first 3–4 mo postgrafting suggest that suppressor cells repopulate the host and mature functionally before helper cells. Whether the B cell or the T helper cell is the target of suppressor cell activity is unclear from these studies. The use of cell free supernatants from T cells or T cell subsets may clarify these questions.

The function of T cells and T cell subsets in blastogenesis experiments from 12 recipients of marrow for aplastic anemia studied within 1 yr from transplant has been explored by other workers. They suggest that poor proliferative response to mitogens, abnormal biochemical properties and distribution of surface markers are a result of immature T cell function. The role of T cell subsets in regulating Ig secretion, on the other hand, will be elucidated when these subsets are studied in the biosynthesis of Ig secretion.

Other workers have investigated in vitro antibody production in a small number of patients between 2 mo and 2 yr postgrafting. Their findings also indicate various T and B cell abnormalities in the lymphocytes of patients after marrow grafting. Of particular note was that the non–T fraction that had failed to secrete immunoglobulin after PWM stimulation was able to secrete Ig when stimulated with Epstein-Barr virus. These findings suggest that B cells are capable of responding to different signals to become active antibody secreting cells. Since most patients recover and become long-term survivors, other nonimmunologic mechanisms such as antibiotics, granulocyte transfusions and treatment in laminar air flow rooms must in

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**Table 2. Qualitative Lymphocyte Function and Serum Immunoglobulin Levels in 16 Patients**

<table>
<thead>
<tr>
<th>UPN*</th>
<th>GVHD status</th>
<th>Day of Ig level</th>
<th>IgG (610–1530)</th>
<th>IgA (70–435)</th>
<th>IgM (38–220)</th>
<th>Day of in vitro test</th>
<th>Failure of</th>
<th>T suppressor activity</th>
<th>Radiosensitivity of T suppressor activity</th>
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<td>1125</td>
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<td>30</td>
<td>925</td>
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<td>1126</td>
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<td>59</td>
<td>832</td>
<td>53</td>
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<td>12</td>
<td>60</td>
<td>+</td>
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</table>

*Unique Patient Number.

Day post grafting.

Failure defined as <5th percentile of distribution of normal function described in the text.

Values lower than normal are italicized.

Denotes when mononuclear cells were cultured with normal T and/or non-T cells because cell numbers yielded from patients were too small to do separations. In these instances the lack of function can be ascribed only to the mononuclear population and not to a subset.

" + " denotes finding present. " - " denotes finding absent.

nd denotes test not done.

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part protect the patients from infections. Other protective mechanisms could include persistence of host immunity for 1–3 mo postgrafting or the passive transfer of mature immunocompetent donor lymphocytes in the marrow infusion which are capable of responding to pathogens.9

Suppressor cells have been described in long-term survivors with chronic GVHD.15,23,26 Whether these are different types of suppressor cells is unclear, but they do have different targets. One target is the cell responding in blastogenesis to alloantigens, and the other is the constellation of cells which are responsible for Ig secretion after stimulation by a polyclonal activator. The relationship of these cells defined in vitro to in vivo events is unclear. As an example of how in vivo and in vitro data are not necessarily consistent, we recently studied a patient with Wiskott–Aldrich syndrome who received an HLA–identical sibling marrow graft.27 Suppressor T cells were present 2 and 4 mo after grafting but absent by 9 mo. He did not have GVHD and his serum antibody response to bacteriophage returned to the normal range 4 mo postgrafting in spite of the presence of suppressor T cells during immunologic reconstitution. These findings suggest that the nonspecific suppressor T cells identified by culture with the PWM during the first 3–4 mo after grafting play a role in regulating an orderly process of immunologic reconstitution rather than suppressing response to specific antigens.

Finally, suppressor T cells have been suggested by some as important in the etiology of hypogammaglobulinemia in congenital immunodeficiency syndromes and in the early months after marrow transplantation for aplastic anemia or hematologic malignancy.22,28 Poor in vitro Ig secretion resulting from the entire spectrum of T and B cell abnormalities is suggested by these data because the polyclonal antihuman Ig, which in our laboratories identified α, μ and γ determinants, did not pick up plaques. Confirmation of this hypothesis can be obtained from experiments where class specific antisera are used. In any case, no apparent relationship was seen in this study between serum Ig levels and in vitro results (Table 2). In contrast to the situation in congenital immunodeficiencies, a plausible explanation for marrow graft recipients is that recipient serum Ig levels in the early postgraft period may not yet result from the function of the engrafted donor stem cells but rather may result from residual host Ig and/or donor Ig infused with the marrow inoculum.9

The remarkably similar function of lymphocyte subpopulations and the similarity of in vivo antibody responses to specific antigens9,17,18 among syngeneic and allogeneic recipients with and without GVHD during the first 3–4 mo after grafting suggest that varying degrees of identity at the histocompatibility loci are not of primary importance for these immunologic functions. This problem points out the need to develop other tests which are specific enough to pick out these differences.

The similarity of function among the patient groups seen in the first 3–4 mo postgrafting leaves no explanation for the differences in infection among syngeneic or allogeneic recipients. Other immunologic mechanisms may be involved in the protection against infection. Our in vitro tests may not be specific enough to detect differences in host resistance to infection. Peripheral blood lymphocyte functions may not be representative of other important lymphoid function operative in tissues such as lymph node, Peyers patches, skin, or lung. Beyond 6 mo, immunologic function of peripheral blood is consistent with clinical differences in infection. Long-term survivors have normal immunologic function and no or very few infections.9,15,29 However, patients with chronic GVHD have immunologic impairment and have a higher incidence of fatal bacterial infection.9,15,29 It seems that the major determinant of the pace of immune reconstitution during the early postgrafting period is the time required for donor cells to repopulate host tissues and to become functionally mature.

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REFERENCES

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