Lymphocyte Dysfunction in Chronic Graft-Versus-Host Disease

By Andrew Saxon, Rosemary E. McIntyre, Ronald H. Stevens, and Robert Peter Gale

Three recipients of HLA-identical bone marrow transplants developed chronic graft-versus-host disease (cGVHD) and hypergammaglobulinemia. All three had evidence of abnormal B-lymphocyte function, including a polyclonal increase in immunoglobulins (Igs), antinuclear antibodies, rheumatoid factor, lymphocytotoxins, and increased immune complexes. T-lymphocyte function was also abnormal, including decreased mitogen reactivity and delayed cutaneous hypersensitivity. The cellular basis of these immune abnormalities was studied in an in vitro system in which we analyzed spontaneous pokeweed mitogen (PWM) driven Ig synthesis. Multiple defects in both T- and B-lymphocyte function were detected. In contrast to normal B cells, circulating B cells from all three patients with cGVHD spontaneously synthesized in vitro >200 ng of IgG and in two of the three >175 ng of IgM. This increase in spontaneous Ig synthesis was not due to a deficiency of regulatory cells, since T cells from the three patients suppressed spontaneous Ig synthesis in a normal fashion. In contrast to this increased spontaneous Ig synthesis, the response of the patients' B cells to PWM-driven Ig synthesis was normal. Using the PWM system we demonstrated several defects in these patients' T cells, including increased suppressor activity and decreased helper cell activity. These data indicate that some patients with cGVHD have multiple defects in both T- and B-cell function that may contribute to their profound immune deficiency.

CASE REPORTS

0106

This 18-yr-old man developed aplastic anemia in February 1978. He failed to improve with conventional therapy. In April 1978, after conditioning with cyclophosphamide and low-dose total body radiation (3 Gy), he received a bone marrow transplant from his HLA-A, B, C, and D matched male sibling. Posttransplant, he received methotrexate (10 mg/sqm) weekly for 102 days to modify GVHD. Hematologic engraftment occurred in the ensuing 3 wk. Four weeks posttransplant, the patient developed a maculopapular rash, liver function abnormalities, and diarrhea. Transient improvement occurred following treatment with corticosteroids and L-asparaginase. Two months posttransplant, the patient developed CMV-related interstitial pneumonitis; he recovered from this without specific therapy. Three months posttransplant, the patient developed cutaneous herpes zoster, which did not disseminate and also resolved. Seven months posttransplant, the patient developed an erythematous rash involving the face, back, and extremities; there was progressive tightening of the skin and dermal atrophy. An inflammatory nondeforming arthritis of the hands and slight hematonegaly were also noted. Liver function tests revealed elevations of alanine aminotransferase, asparate aminotransferase, and alkaline phosphatase. The bilirubin was normal. The patient is more than 3 yr posttransplant with normal bone marrow function and moderately severe chronic GVHD. His circulating immune complexes are twice the upper limit of normal, and he has lymphocytotoxins to greater than 80% of random normal lymphocytes. Antinuclear antibody was positive at 1:10240 with a homogeneous and speckled pattern and a rheumatoid factor positive at 1:312.

0081

This 10-yr-old female developed acute lymphoblastic leukemia in March 1974. She was treated with conventional therapy but had multiple bone marrow relapses. In June 1977 while in her fourth remission, she received a bone marrow transplant from her HLA-A, B, C, and D identical sister. The pretransplant conditioning regimen consisted of doxorubicin (60 mg/sqm) and fractionated total body radiation (11.4 Gy). Because the donor and recipient were ABO incompatible (donor AB, recipient A), phasemapheresis was performed immediately prior to the transplant. Methotrexate (10 mg/sqm) was given weekly after transplant for 102 days to modify GVHD. Engraftment occurred by the third week. One week later, the patient developed a generalized rash and a modest elevation of...
serum alanine aminotransferase. A skin biopsy was consistent with mild GVHD. These abnormalities resolved over the next 2 wk. Five months later, the patient developed skin changes suggestive of scleroderma with hide-bound skin and flexion contractures. A skin biopsy revealed dermal fibrosis. Her skin lesions progressed over the next 6 mo. She developed hypergammaglobulinemia. She received a short-course of melphalan therapy without improvement. Over the next year the skin lesions stabilized and IgG levels slowly declined toward normal. Pulmonary function tests revealed mild restrictive lung disease. Two years posttransplant, she developed cutaneous herpes varicella without significant sequelae. She is now 3.75 yr after transplantation. She too has elevated circulating immune complexes (2.5 times normal) as well as antibodies to DNA and IgG.

0184

This 40-yr-old man developed acute myelogenous leukemia in July 1980. He was treated with a single course of cytarabine, daunorubicin, and 6-thioguanine and achieved a remission. In October 1980, he received a bone marrow transplant from his HLA-A, B, C, and D identical sibling after conditioning with cyclophosphamide (120 mg/kg) plus total body radiation (10 Gy). Methotrexate (10 mg/sqm) was given weekly posttransplant for 102 days to modify GVHD. Marrow engraftment occurred in the 3 wk following transplant. He developed a diffuse rash 6 wk posttransplant, which was treated with prednisone (100 mg/day for 7 days) with considerable improvement. The patient first noted tightness of the skin, while early dermal fibrosis was evident on physical exam 2 wk later. He received prednisone (60 mg every other day) and azathioprine (50 mg/sqm/day). Five and one-half months posttransplant the patient developed disseminated varicella zoster infection, which was fatal.

MATERIALS AND METHODS

Lymphocyte Preparation

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque density gradient centrifugation of heparinized blood from the patients and normal volunteers, aged 25–35 yr. T lymphocytes were enumerated by rosette formation with sheep erythrocytes (E-rosettes) and B lymphocytes by direct staining of surface immunoglobulin (SmIg) with a polyvalent conjugated goat anti-human immunoglobulin. Cells with complement receptors (C') were detected by rosette formation with antibody and complement-coated sheep erythrocytes. These techniques have been reported in detail.23 T- and B-lymphocyte-enriched fractions were prepared by density centrifugation of PBMC after formation of rosettes with sheep erythrocytes modified by 2-amino isothiouronium bromide hydrobromide.24 T-lymphocyte-enriched fractions contained greater than 90% E-rosette-forming cells and less than 2% SmIg-positive cells. The T-lymphocyte-depleted (B-lymphocyte) fraction contained 65%-80% SmIg-positive cells, 20%-35% phagocytic cells, and less than 5% E-rosette-forming cells.

Immune Studies

Serum levels of IgG, IgM, and IgA were determined by laser nephelometry (Hyland Labs, Costa Mesa, Calif.). Complement-fixing antibodies to CMV and toxoplasma were determined by standard techniques. Standard serologic assay of rheumatoid factor, antinuclear antibodies, and complement were performed. Immune complexes were determined by precipitation with polyethylene glycol.23 Mitogen responsiveness to phytohemagglutinin (PHA) and concanavalin A (Con-A) were studied using a semimicro technique.26 Briefly, PBMC were incubated with PHA (1:10–1:1000) (Difco Labs, Detroit, Mich.) or Con-A (Calbiochem, Los Angeles, Calif.) for 72 hr, pulsed with tritiated thymidine (3HTdR, New England Nucleic, Boston, Mass.) for 16 hr, and 3HTdR incorporation determined by liquid scintillation counting.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Days Posttransplant</th>
<th>IgG</th>
<th>IgM</th>
<th>IgM % SmIg</th>
<th>% EA*</th>
<th>% T*</th>
<th>PHA§</th>
<th>Con-A§</th>
<th>DCH‡</th>
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<td>300</td>
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<td>15</td>
<td>82</td>
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<td>130</td>
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<td>40</td>
<td>3</td>
<td>15</td>
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</table>

*Percent B cells by surface membrane immunoglobulin.
†Percent Fcγ receptor cells by binding IgG with red blood cells.
‡Percent T cells by sheep red blood cell rosette assay.
§Ratio of PHA- and Con-A-induced blastogenesis to normal.
‡Delayed cutaneous hypersensitivity to a panel of 5 antigens as described in text.
incubated in a humidified atmosphere at 37°C with 5% CO₂. Supernatants were harvested after 3 and 8 days and analyzed for immunoglobulin (Ig) synthesis. None of the patients were receiving immunosuppressive drugs at the time of in vitro Ig production studies.

Radioimmunoassay
Quantitative radioimmunoassays for in vitro IgG and IgM were performed as previously described. In each radioimmunoassay, a seven-point standard curve was determined and experimental IgG and IgM were calculated from the curve.

RESULTS

Immune Studies
Serial determinations of immune function in the patients are summarized in Table 1. The total numbers of T and B lymphocytes rose rapidly following transplantation, however, T cells remained lower than normal in patient 0081. In contrast, mitogen responsiveness and skin test reactivity remained depressed. Coincident with the onset of chronic GVHD, the patients developed features of autoimmunity, including polyclonal hypergammaglobulinemia, elevated titers of rheumatoid factor and antinuclear antibodies, elevated immune complexes, and nonspecific lymphocytotoxins to the panel of unrelated normal cells.

Spontaneous Immunoglobulin (Ig) Synthesis
In vitro spontaneous IgG synthesis by fractionated B lymphocytes of patient 0106 was determined to be 669 ng and 344 ng in two separate experiments (Table 2). In contrast, genetically identical B lymphocytes from the marrow donor and cells from unrelated donors synthesized less than 50 ng of IgG. No detectable IgM was spontaneously synthesized under these conditions, either by this patient or the normals. Patients 0081 and 0184 synthesized 687 ng and 206 ng of IgG, 410 ng and 186 ng of IgM under identical conditions.

We previously reported that a subset of radiation-sensitive T lymphocytes is capable of partially inhibiting spontaneous Ig synthesis by B lymphocytes. We next studied the regulatory function of patient T cells by testing the effect of untreated or irradiated

<table>
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<th>Days Posttransplant</th>
<th>B-Cells</th>
<th>Source of T Lymphocytes</th>
</tr>
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<tr>
<td>335</td>
<td>None</td>
<td>Patient Normal Patient Normal</td>
</tr>
<tr>
<td>425</td>
<td>669</td>
<td>344 218 252 318 324</td>
</tr>
</tbody>
</table>

B cells from the patient (0.4 × 10⁶) were cultured for 3 days alone or with T cells (1.6 × 10⁶) either from the patient or from an unrelated normal (day 426) or from the marrow donor (day 493). Subscript x refers to T cells irradiated with 3000 rads. Results are expressed as ng Ig synthesized per culture. B cells from normals synthesized less than 50 ng of IgG under these conditions.

Therefore, both patient and normal T cells were added to patient B cells to determine whether the patients might lack such suppressor T cells or if patient B cells were insensitive to these normal suppressor cells. Unirradiated T lymphocytes from patient 0106, a normal individual, and the bone marrow donor all decreased spontaneous IgG synthesis by the patient’s B lymphocytes. Irradiation of the normal T lymphocytes abolished this suppressive effect (Table 2). Similar results were obtained in experiments using cells from patient 0081 and 0184 (data not shown).

PWM-Driven Ig Synthesis
We previously reported that B lymphocytes that synthesize Ig following PWM stimulation differ from those that spontaneously synthesize IgG. The PWM-driven response requires the addition of helper T cells, whereas spontaneous Ig synthesis is T-cell independent. PWM stimulation of B lymphocytes from patient 0106 and normal or donor T cells resulted in normal levels of IgG and IgM synthesis (Table 3). Irradiation of the normal T cells resulted in the expected increase (20%-60%) in Ig synthesis. Similar results were obtained with B cells from patient 0184 (Table 3). This study was not performed with cells from patient 0081 because of a limited number of available cells.
LYMPHOCYTE DYSFUNCTION IN CHRONIC GVHD

Table 4. Helper and Suppressor Cell Activities in PWM-Driven Ig Synthesis Using Normal B Lymphocytes

<table>
<thead>
<tr>
<th>Patient</th>
<th>Days After Transplant</th>
<th>Source of T Cells</th>
<th>IgG (ng)</th>
<th>IgM (ng)</th>
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<td>Normal</td>
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<tr>
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<td></td>
<td>Patient</td>
<td>—</td>
<td>—</td>
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<td>+</td>
<td>1,290</td>
<td>2,505</td>
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<td></td>
<td></td>
<td>*</td>
<td>38</td>
<td>75</td>
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<tr>
<td>0106</td>
<td>425</td>
<td>+</td>
<td>3,927</td>
<td>2,813</td>
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<td></td>
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<td>2,614</td>
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<td>1,064</td>
<td>583</td>
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<tr>
<td>0184</td>
<td>120</td>
<td>+</td>
<td>1,963</td>
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<td></td>
<td></td>
<td>*</td>
<td>201</td>
<td>244</td>
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Normal B lymphocytes (0.4 x 10⁶) were cultured 7 days with T lymphocytes (1.6 x 10⁶) from the patient or a normal. In some cultures the T lymphocytes were irradiated (asterisk) with 3000 rads to remove suppressor influences. The normal in the third experiment was the bone marrow donor for patient 0108.

Table 5. Effect of Additional Patient T Lymphocytes on PWM-Driven Ig Synthesis by Normal B and T-Cell Cultures

<table>
<thead>
<tr>
<th>Patient</th>
<th>Days After Transplant</th>
<th>Source of Extra T Cells</th>
<th>IgG (ng)</th>
<th>IgM (ng)</th>
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<td>+</td>
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<td>2,175</td>
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<td></td>
<td></td>
<td>+</td>
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<td>833</td>
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<tr>
<td></td>
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<td>+</td>
<td>1,481</td>
<td>1,080</td>
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<tr>
<td></td>
<td></td>
<td>+</td>
<td>736</td>
<td>625</td>
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<tr>
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<td>2,424</td>
<td>1,672</td>
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<td>+</td>
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<td></td>
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<td>*</td>
<td>906</td>
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B cells (0.4 x 10⁶) from normals were cultured 7 days with additional T cells (0.4 x 10⁶) from the patient or the normal in the presence of PWM, and Ig synthesis was then determined. In some experiments, the additional T cells were irradiated (asterisk) to remove suppressor influences. In the third experiment the normal was the marrow donor for patient 0106.

DISCUSSION

A detailed analysis of immune function in our patients with chronic GVHD and hypergammaglobulinemia revealed a spectrum of T- and B-lymphocyte abnormalities. These selected patients had polyclonal hypergammaglobulinemia. Their B lymphocytes showed increased in vitro spontaneous synthesis of IgG and IgM. These data contrast with those of Ringden et al. who found increased spontaneous IgG plaque-forming cells in acute but not chronic GVHD. Increased spontaneous IgG synthesis has also been reported in patients with systemic lupus erythematosus and in normals following booster immunization. The continued presence of these activated lymphoblastoid B cells in our patients suggest an ongoing humoral response by the donor cells. This response could be to recipient antigens, as the patients had GVHD and impaired humoral responses to exogenous antigens, but the cells may have been reacting toward a variety of antigen stimuli.

T cells on PWM-driven Ig production by normal B lymphocytes (Table 4). Unirradiated T cells from the patients were less effective in promoting PWM-driven Ig synthesis than normal T cells. In patients 0106 and 0184, irradiation of the patient T cells resulted in increased PWM-driven Ig synthesis by normal B cells compared to the unirradiated T cells. The percent increase of IgG and IgM synthesis following irradiation of the patient T cells was greater than that observed following irradiation of T cells from normals; however, total Ig production remained depressed compared to equivalent normal cultures.

These results can be explained by either excessive activity by radiosensitive suppressor T cells, or by a quantitative deficiency of helper T-cell activity. To evaluate these possibilities, we tested nonirradiated and irradiated T cells from all three patients for their effect on the PWM-driven Ig synthesis in cultures of B and T lymphocytes from normals (Table 5). In these experiments, the normal T cells were unirradiated so as to provide a constant level of helper T-cell activity as well as radiosensitive amplifier cells necessary for the expression of T-cell-mediated suppression. Addition of unirradiated patient T cells markedly inhibited IgG and IgM synthesis on contrast to cultures with extra untreated normal cells. Compared to normal cultures, this suppression was not observed when the patient T cells were irradiated. However, Ig synthesis produced by cultures containing additional irradiated patient T cells were consistently lower than analogous cultures containing irradiated normal T cells (Table 5).
Levels of spontaneous IgG synthesis detected in chronic GVHD patients are comparable to those observed in normal 6 days following tetanus immunization. These B cells appear to reflect an ongoing immune response and its dissemination as evidenced by the occurrence at the time of appearance of antibody. We have found that repeated immunization results in inhibition of spontaneous in vitro Ig antibody synthesis, and continuous antigen stimulation of the donor cells in vivo may account for the fact that this spontaneous IgG production was not even greater.

We also reported that a radiosensitive subset of peripheral blood T cells can inhibit IgG synthesis by lymphoblastoid B cells. The activity of this subset appears to be normal in our patients, and thus, we cannot ascribe the observed increased Ig synthesis to a loss of regulatory T-cell activity, at least as measured in the circulation. Of interest was our ability to detect spontaneous synthesis of IgM in vitro in two of the three patients. Similar data have been reported in patients with dysgammaglobulinemia type I who have elevated levels of IgM and depressed levels of IgG and IgA. Spontaneous IgM antibody production in vitro was not observed by us following booster immunization.

In a separate series of experiments we studied PWM-driven B-cell synthesis of IgG and IgM in these patients. This proved to be normal. This is consistent with data by Ringden and coworkers who found normal plaque-forming cells to Staphylococcus aureus in long-term survivors of marrow transplantation. We also reported normal PWM-driven total Ig synthesis in 2 transplant patients studied greater than 100 days posttransplant who did not have GVHD. In the PWM system, IgG but not IgM synthesis requires a recent exposure to antigen. Since in vitro patient B cells could synthesize both PWM-driven IgG and IgM, one might predict, in the absence of other defects, normal antibody responses in vivo as these cells appear to represent a population of memory B cells. Most clinical data are to the contrary.

Analysis of regulatory T cells in our patients revealed two distinct defects. First, they had excessive suppressor T cell activity capable of inhibiting Ig synthesis by autologous B cells, B cells from the marrow donor, and B cells from unrelated normals. In contrast to Reinherz and colleagues, who reported activated Th2, Ia+ suppressor and/or cytotoxic T cells in two patients with chronic GVHD, we did not find evidence for activated radiation-resistant suppressor T cells as irradiation removed the inhibitory activity of the patient T cells. However, both of their patients had hypogammaglobulinemia. They also failed to find radiosensitive suppressor cells in patients with normal to increased levels of immunoglobulins. We have studied 2 additional long-term bone marrow transplantation survivors without GVHD; neither had excessive T-suppressor cell activity.

It may appear contradictory to find patients with hypergammaglobulinemia who have excessive suppressor T lymphocytes. Recent studies, however, have reported that only a small percentage of B cells respond to PWM and these represent cells in a functional state of maturation of specific antibody responsiveness that differs for IgM and IgG. Thus, T cells that inhibit PWM responsiveness are capable of inhibiting specific B-cell antibody responses, responses reported to be abnormal in transplant patients with GVHD.

A second regulatory defect in our patients was a relative lack of T helper cells. Although irradiated T cells of the patients failed to suppress Ig synthesis by normals, they did not provide normal levels of T-cell help for IgG and IgM synthesis by B lymphocytes.

Based on our results we postulate that polyclonal hypergammaglobulinemia, autoantibodies, and immune complexes in some patients with chronic GVHD are generated during a period of relative T-cell imbalance and immunosuppression that follows bone marrow transplantation. This induces the compensatory activation of suppressor T cells, which is reflected in the T-cell-mediated suppression of PWM-driven Ig synthesis. Whether chronic GVHD induces these immune abnormalities or whether the immune imbalance produced chronic GVHD is presently unknown.

ACKNOWLEDGMENT

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LYMPHOCYTE DYSFUNCTION IN CHRONIC CVHD


Lymphocyte dysfunction in chronic graft-versus-host disease

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