Pokeweed Mitogen–induced B Cell Differentiation in Idiopathic Aplastic Anemia Associated With Hypogammaglobulinemia

By Takashi Uchiyama, Kenichi Nagai, Morihisa Yamagishi, Kiyoshi Takatsuki, and Haruto Uchino

Peripheral blood lymphocytes from 14 patients with idiopathic aplastic anemia were examined to determine their capacity to differentiate into immunoglobulin (Ig)-producing cells in the presence of pokeweed mitogen (PWM). Four patients had hypogammaglobulinemia. Lymphocytes from ten patients without and one with hypogammaglobulinemia were capable of differentiating into Ig-producing cells. Lymphocytes from three patients with hypogammaglobulinemia, however, were unable to differentiate. Coculture experiments showed that in one patient helper activity of T cells was impaired, in another patient B cells could not be stimulated by PWM to differentiate even in the presence of normal T cells, and in a third patient both the helper function of T cells and the capacity of B cells to differentiate into Ig-producing cells were affected. These results suggest that differentiation of lymphoid cells is impaired in some cases of idiopathic aplastic anemia associated with hypogammaglobulinemia.

IDIOPATHIC APLASTIC ANEMIA (AA) is a disorder characterized by peripheral blood pancytopenia and hypocellular bone marrow. The pathogenesis of the disease remains obscure, although hypothetical mechanisms such as the abnormality of hematopoietic pluripotent stem cells and environmental factors that fail to support normal hematopoiesis have been postulated. Marked decrease in erythrocytes, granulocytes, and platelets, impaired colony formation in vitro, and successful bone marrow grafting in aplastic anemia strongly suggested that erythroid, myeloid, and megakaryocytoid stem cells are impaired. Production and differentiation of lymphocytes have not been considered impaired, and both immunoglobulin (Ig) production and delayed hypersensitivity are usually normal. Some cases of AA, however, have been reported to be associated with hypogammaglobulinemia. Differentiation of lymphoid cells may also be impaired in such cases.

Pokeweed mitogen (PWM) has been reported to induce B cell differentiation into Ig-producing cells with the aid of the helper function of normal T cells. Mechanisms of immunodeficiencies in diseases such as common variable hypogammaglobulinemia, multiple myeloma, and systemic lupus erythematosus have been investigated using this model in vitro for B cell differentiation. We studied PWM-induced differentiation into Ig-producing cells of peripheral blood lymphocytes from four patients with idiopathic AA associated with hypogammaglobulinemia and ten patients without it.
MATERIALS AND METHODS

Patients. Fourteen patients with AA were studied. The diagnosis of idiopathic AA was made when all of the following criteria were fulfilled: peripheral blood pancytopenia (RBC less than 3.5 x 10¹²/liter, WBC less than 4.0 x 10⁹/liter, platelet count less than 100 x 10⁹/liter); hypoplastic bone marrow disclosed by bone marrow aspiration and/or bone marrow biopsy; no other disorders such as leukemia, paroxysmal nocturnal hemoglobinuria, or systemic lupus erythematosus that can cause pancytopenia; and no exposure to etiologic agents such as drugs, chemicals, and radiation that can cause pancytopenia. Data on four patients with AA associated with hypogammaglobulinemia are summarized in Table 1. All four patients had received multiple blood transfusions at least 3 mo before our studies. Small doses (5 mg/day) of prednisolone had been given to patients H.Y., K.Y., and N.K., in addition to large doses (105 mg/day) of oxymetholone to patient N.K. These drugs were withdrawn at least 2 wk before the study. The ten patients without hypogammaglobulinemia included five males and five females, ages 28-62 yr. All ten patients had been given blood transfusions. At the time of these studies, five patients were being treated with anabolic steroids and adrenal corticosteroids, four with only anabolic steroids, and one without any drugs.

Separation of peripheral blood lymphocytes. Peripheral blood lymphocytes were separated by Ficoll-sodium metrizoate gradient centrifugation.¹⁴ The method of separation of T cells (T-enriched cell population) and B cells (T-depleted cell population) by sheep red blood cell (E) rosette centrifugation through Ficoll sodium metrizoate gradient and lysis of attached E by addition of 0.83% Tris buffered ammonium chloride solution was described elsewhere.¹⁵ We found that 90%-95% of the T-enriched cell population were E rosette-forming cells, and surface immunoglobulin (SIg)-bearing cells comprised less than 0.3%. The T-depleted cell population consisted of SIg-bearing cells (39%-45%), E rosette-forming cells (<4%), and monocytes.

Cell surface markers. E rosette forming cells were detected by a slight modification of the method described by Jondal et al.¹⁶ SIg-bearing cells were studied by a direct immunofluorescence technique in ice with fluorescein isothiocyanate (FITC)-conjugated anti-human Ig (Behringwerke, Marburg Lahn, W. Germany), which had been absorbed with human erythrocytes before staining. Monocytes present in mononuclear cell suspensions separated from peripheral blood were identified microscopically in peroxidase-stained cytocentrifuge preparations.

Receptors for the Fc portion of IgG and for IgM were detected by rosette formation with ox red blood cells (ORBC) heavily coated with purified rabbit IgG or IgM anti-ORBC.¹⁷ IgG and IgM antibodies were isolated by DEAE cellulose column chromatography and Sephadex G-200 column chromatography and were confirmed to give a single precipitation line by immunoelectrophoresis. Equal volumes of 2.0 x 10⁹/ml T cells (T-enriched cell population) in RPMI-1640 medium supplemented with 20% fetal calf serum (FCS) (Grand Island Biological, Grand Island, N.Y.) and 1% ORBC sensitized with IgG or IgM antibodies were mixed, centrifuged at 150 g for 5 min and incubated at room temperature (20°C) for 60 min. The resulting pellet was gently resuspended and put into a hemocytometer chamber. Lymphocytes with three or more adherent red cells were counted as rosette-forming cells. Receptors for IgM were studied on T cells cultured in RPMI-1640 supplemented with 20%, FCS for 15 hr.

Table 1. Summary of Four Patients With Idiopathic Aplastic Anemia and Hypogammaglobulinemia

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>RBC (x 10¹²/liter)</th>
<th>WBC (x 10⁹/liter)</th>
<th>Lymphocytes (%)</th>
<th>Platelets (x 10⁹/liter)</th>
<th>Serum Ig (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H.Y.</td>
<td>30M</td>
<td>2.28</td>
<td>3.0</td>
<td>72</td>
<td>14.0</td>
<td>490</td>
</tr>
<tr>
<td>K.K.</td>
<td>53F</td>
<td>1.57</td>
<td>2.2</td>
<td>50</td>
<td>70.0</td>
<td>480</td>
</tr>
<tr>
<td>K.Y.</td>
<td>21M</td>
<td>1.71</td>
<td>1.7</td>
<td>61</td>
<td>20.0</td>
<td>740</td>
</tr>
<tr>
<td>N.K.</td>
<td>31M</td>
<td>1.67</td>
<td>1.9</td>
<td>44</td>
<td>28.0</td>
<td>575</td>
</tr>
</tbody>
</table>

Aplastic anemia without hypogammaglobulinemia (average ± SD, n = 10) 1390 ± 231 232 ± 113 144 ± 126
Normal (average ± SD, n = 20) 1398 ± 251 191 ± 73 127 ± 64 (male) 179 ± 96 (female)
Cell culture and immunofluorescence. Methods of detection of cytoplasmic Ig-positive cells in PWM-stimulated lymphocytes were as described elsewhere. In brief, 1 ml of 1.0 x 10⁶/ml lymphocytes was cultured in a multidish tray (Linbro Scientific, Hamden, Conn.) in RPMI-1640 supplemented with 20% FCS and 50 µg/ml kanamycin in the presence of an optimal dose of PWM (Gibco, Grand Island, N.Y.) in a humid atmosphere of 5% CO₂ for 7 days. On day 4 half the culture medium was replaced by fresh medium. In coculture experiments, equal numbers of peripheral blood lymphocytes from a normal individual and peripheral blood lymphocytes or T cells (T-enriched cell population) from a patient with idiopathic AA, or 8.0 x 10⁵ T cells (T-depleted cell population) and 2.0 x 10⁵ B cells (T-depleted cell population), were cocultured. In every culture final cell concentration was adjusted to 1.0 x 10⁶/ml. On day 7 numbers of recovered cells and viability, determined by the trypan blue dye exclusion test, were checked. Cytocentrifuge preparations were fixed in 95% cold ethanol for 15 min and stained with FITC-conjugated antihuman immunoglobulins (Dako, Copenhagen, Denmark) that had been absorbed with human erythrocytes and filtered through a Millipore filter. The cells positive for cytoplasmic Ig were counted. Cytoplasmic Ig-positive cells generated from 10⁶ lymphocytes stimulated by PWM were calculated as follows: total cell count recovered x viability (%) x cytoplasmic Ig-positive cells (%) x 100. In order to determine suppressive or enhancing effect, the ratio of cytoplasmic Ig-positive cells found to those expected on the basis of dilution alone (found/expected value, F/E) was calculated as described elsewhere.

Measurement of serum Ig levels. Serum IgG, IgA, and IgM levels were determined by the single radial immunodiffusion method using an antiserum-containing agar plate prepared in our laboratory. The antisera were obtained from rabbits immunized with normal IgG, myeloma IgA, and primary macroglobulinemia IgM, and their specificity was confirmed by immunoelectrophoresis after appropriate absorptions.

RESULTS

Serum Ig levels. As shown in Table 1, serum IgG levels of all four patients with hypogammaglobulinemia, IgA of patient K.K., and IgM of patients H.Y. and K.K. were much lower than normals, although these values were not as low as those usually seen in primary immunodeficiencies. In patients H.Y., K.K., and N.K., such low serum IgG levels as shown in Table I had persisted since the onset of the disease. The serum IgG level of patient K.Y. had been about 900 mg/dl at the onset of the disease and had been decreasing. Serum Ig levels of ten other patients were not lower than normal.

Lymphocyte subpopulations, T cell subsets, and PWM-induced B cell differentiation. Lymphocyte subpopulations, T cell subsets and cytoplasmic Ig-positive cells generated from PWM-stimulated lymphocytes in patients with AA and normal individuals are shown in Table 2. The percentage of E rosette-forming cells (T) was higher than normal in patients H.Y., K.K., and N.K. T cells having receptors for IgM (Tµ) were much fewer in patient K.K., and T cells having receptors for IgG (Tγ) were higher in patients H.Y., K.Y., and especially K.K. SIg-bearing cells were slightly fewer than normal in patients K.K. and N.K. Ten patients without hypogammaglobulinemia showed almost normal values of E rosette-forming and SIg-bearing cells.

The number of cytoplasmic Ig-positive cells generated from PWM-stimulated lymphocytes from patient H.Y. was slightly below the lower limit of normals, whereas those numbers in the other three patients with hypogammaglobulinemia (K.K., K.Y., and N.K.) were extremely low. The peripheral blood lymphocytes from the ten patients without hypogammaglobulinemia, however, were found to differentiate normally into cytoplasmic Ig-positive cells in the presence of PWM.
Table 2. Lymphocyte Subpopulations, T Cell Subsets, and PWM-induced B Cell Differentiation in Four Patients With Idiopathic Aplastic Anemia and Hypogammaglobulinemia

<table>
<thead>
<tr>
<th>Patient</th>
<th>T (%)</th>
<th>T(+)</th>
<th>T(+)</th>
<th>Slg(+)</th>
<th>Clg-positive Cells (x 10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H.Y.</td>
<td>84.4</td>
<td>41.4</td>
<td>16.7</td>
<td>6.9</td>
<td>0.38</td>
</tr>
<tr>
<td>K.K.</td>
<td>97.1</td>
<td>17.0</td>
<td>46.4</td>
<td>4.1</td>
<td>0.03</td>
</tr>
<tr>
<td>K.Y.</td>
<td>77.9</td>
<td>42.7</td>
<td>13.0</td>
<td>12.9</td>
<td>0</td>
</tr>
<tr>
<td>N.K.</td>
<td>84.0</td>
<td>55.2</td>
<td>10.0</td>
<td>4.3</td>
<td>0</td>
</tr>
</tbody>
</table>

Aplastic anemia without hypogammaglobulinemia
(average ± SD, n = 10)

| Normal  | 64.2 ± 13.2 | 9.0 ± 4.9 | 1.43 ± 0.90 |

T, E rosette-forming cells; T(+) cells having receptors for IgM; T(+) cells having receptors for IgG; Slg, surface Ig-bearing cells; Clg, cytoplasmic Ig-positive cells generated from 1.0 x 10^6 lymphocytes in the presence of pokeweed mitogen.

*Percentage of peripheral blood lymphocytes (not including monocytes).
†Percentage of T cells.

Cocultures of lymphocytes from patients and normals. In order to analyze the mechanisms of the failure of PWM-induced lymphocyte differentiation, coculture experiments of peripheral blood lymphocytes and T and B cells from four patients with AA associated with hypogammaglobulinemia and those from normal individuals were performed. Results are shown in Fig. 1. Differentiation of peripheral blood lymphocytes from patient H.Y. was rather poor as compared with that of lymphocytes from normal subjects, but when T

Fig. 1. PWM-induced B cell differentiation into Ig-producing cells in cocultures of lymphocytes from four patients with AA associated with hypogammaglobulinemia and from normal individuals. 10^6 peripheral blood lymphocytes unseparated into T (T enriched) and B (T depleted) fractions (U) were cultured or 8.0 x 10^5 T cells and 2.0 x 10^5 B cells, equal numbers (5.0 x 10^5) of unseparated lymphocytes from a patient and a normal subject, or equal numbers (5.0 x 10^5) of unseparated lymphocytes and T cells were cocultured, for 7 days in the presence of PWM. In these experiments lymphocytes were not cultured in duplicate because of limitations of available cell numbers. Studies repeated in the same individuals at different times, however, showed a good reproducibility. Pt, patient; N, normal subject; ND, not done. Dotted lines indicate values of cytoplasmic Ig-positive cells predicted on the basis of dilution alone in cocultures.
cells (T-enriched cell population) and B cells (T-depleted cell population) separated by E rosette centrifugation through Ficoll-sodium metrizoate gradient were mixed at the ratio of 4:1 (determined to be optimal in normal B cell differentiation) and were cultured in the presence of PWM a much greater number of cytoplasmic Ig-positive cells was generated.

Results of other cocultures indicated that both the helper function of T cells and the capacity of B cells to differentiate into Ig-producing cells were normal. Differentiation of lymphocytes from patient K.K. was extremely poor even when T and B cells were mixed at the optimal ratio and cultured. B cells from patient K.K. differentiated well into Ig-producing cells with the aid of T cells from a normal subject. The helper effect of T cells from patient K.K., however, was poor as compared with that of normal T cells. Coculture of lymphocytes from patient K.K. and normal subject generated 284% of the number of cytoplasmic Ig-positive cells expected on the basis of dilution alone. B cells from patient K.Y. did not differentiate even in the presence of normal T cells, whereas T cells were found to show a normal helper effect on normal B cell differentiation. B cells from patient N.K. also did not differentiate into Ig-producing cells even in the presence of normal T cells, and T cells were not able to permit normal B cells to differentiate into Ig-producing cells.

As reported previously, neither normal peripheral blood lymphocytes nor normal T cells show a significant suppressive effect on PWM-induced differentiation of allogeneic normal B cells. In 21 cocultures of peripheral blood lymphocytes from pairs of normal subjects or peripheral blood lymphocytes and allogeneic T cells at 1:1 cell mixtures, $F/E$ was $1.4 \pm 0.56$ (mean $\pm$ SD) (range 0.64–2.84). T cells from patient N.K., however, suppressed (by 79%) $F/E = 0.21$) normal peripheral blood lymphocyte differentiation in coculture experiments.

Helper activity of T cells, differentiation capacity of B cells, and a suppressive effect on normal B cell differentiation of lymphocytes from four patients are summarized in Table 3.

### DISCUSSION

Peripheral blood lymphocytes from three of four patients with idiopathic AA associated with hypogammaglobulinemia were unable to differentiate into Ig-producing cells in the presence of PWM, whereas those from ten patients with AA not associated with hypogammaglobulinemia did differentiate normally. It seems most unlikely that abnormal findings in patients with hypogammaglobulinemia were due to the effect of treatment because lymphocytes from ten

<table>
<thead>
<tr>
<th>Patient</th>
<th>Helper Function of T Cells</th>
<th>Differentiation of B Cells</th>
<th>Suppressive Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>H.Y.</td>
<td>Normal</td>
<td>Normal</td>
<td>No</td>
</tr>
<tr>
<td>K.K.</td>
<td>Impaired</td>
<td>Normal</td>
<td>No</td>
</tr>
<tr>
<td>K.Y.</td>
<td>Normal</td>
<td>Impaired</td>
<td>No</td>
</tr>
<tr>
<td>N.K.</td>
<td>Impaired</td>
<td>Impaired</td>
<td>Yes</td>
</tr>
</tbody>
</table>
patients also treated with the same drugs responded normally to PWM and differentiated well into Ig-producing cells.

Severe monocytopenia, which has been reported to be associated with aplastic anemia in some cases, did not seem to contribute to the impairment of PWM-induced B cell differentiation in our cases because good numbers of monocytes (6.4%, 7.1%, 8.3%, and 5.5% of mononuclear cells separated from peripheral blood from patients H.Y., K.K., K.Y., and N.K., respectively) were present in each culture.

Coculture experiments showed the defects in lymphocyte differentiation in these three cases to be heterogeneous. In patient K.K. helper activity of T cells was impaired and cell surface marker analysis showed an increase in proportion of T\(\gamma\) and a decrease in T\(\mu\). Recently, Moretta et al. reported that in a T cell dependent model in vitro for B cell differentiation induced by PWM, T\(\mu\) helped and T\(\gamma\) suppressed B cell differentiation. In this case, the impairment of lymphocyte differentiation may be ascribed to the abnormality of the relative proportion of T\(\mu\) and T\(\gamma\) rather than to the intrinsic defect of T cells. In patients K.Y. and N.K., however, peripheral blood B cells were not capable of differentiating into Ig-producing cells even in the presence of the helper activity of normal T cells. In AA it is generally believed that differentiation of lymphoid cells is not severely impaired, but results in these two patients strongly suggest that in some cases of AA associated with hypogammaglobulinemia there is an impairment of differentiation of lymphocytes.

It is also noteworthy that T cells from patient N.K. were found to suppress differentiation of normal B cells. It is possible that the failure of T cells from patient N.K. to exert helper activity in cocultures with normal B cells was due to excessive suppressor T cell function that masked residual normal helper function. In preliminary experiments, in an attempt to examine this possibility T cells from patient N.K. x-irradiated (2000 R) prior to coculture were also seen not to permit normal B cells to differentiate into Ig-producing cells (data not shown). In this model for B cell differentiation, helper activity of T cells was resistant to irradiation, whereas suppressor function of T cells was sensitive to irradiation. Accordingly, it is most likely that T cells of patient N.K. lacked helper function and also had excessive suppressor function in this experimental model.

Recently, two groups reported that lymphocytes from patients with AA suppressed erythroid or myeloid colony formation, and Litwin and Zanjani showed that lymphocytes from two patients with immunodeficiency and with thymoma, one of whom had repeated episodes of red blood cell agenesis, suppressed both PWM-induced B cell differentiation and erythroid differentiation. Although the presence of such suppressor cells might be a consequence of disordered hematopoiesis in AA, it appears more likely that suppressor cells play an important role in the pathogenesis of this disease at least in some patients. Studies are underway in an attempt to determine if T cells from patient N.K. could also suppress erythroid or myeloid colony formation.

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