Correlation of Response of Aplastic Anemia Patients to Antilymphocyte Globulin With In Vitro Lymphocyte Stimulatory Effect: Predictive Value of In Vitro Test for Clinical Response

By Tokuichiro Abe, Hiroshi Matsuoka, Seiji Kojima, Yoshiro Kamachi, Ikuya Tsuge, Yoshihisa Kodera, and Takaharu Matsuyama

Therapy with antilymphocyte globulin (ALG) has been shown to be effective in restoring hematopoiesis to some patients with aplastic anemia. It would be useful to have a method for predicting those likely to be responders versus nonresponders. The mode of immunostimulatory action of ALG is of interest in addition to its immunosuppressive action. We examined in vitro the distribution of the proliferative responses of ALG-stimulated peripheral blood mononuclear cells (PBMCs) obtained from 18 patients with aplastic anemia, eight of whom responded to ALG and 10 who did not. We found a significant difference in the proliferative response of PBMCs obtained from the eight responders versus the 10 nonresponders ($P < .01$). Two-color flow cytometry analysis of the patients' PBMCs stimulated by ALG in vitro showed that the CD4-positive subsets were activated to a greater extent by ALG than the CD8-positive subsets. Moreover, a positive correlation with the clinical response of patients to ALG with granulocyte-macrophage colony-stimulating factor produced by their PBMCs stimulated by ALG suggests that the immunostimulatory property of ALG has an important role in the treatment of aplastic anemia. Our results suggest that the clinical response to ALG therapy is correlated with its lymphocyte proliferative effect in vitro, and indicates that the assessment of the proliferative response of PBMCs in vitro would be useful in predicting the clinical response to ALG therapy.

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A LTHOUGH THE CLINICAL RESPONSE varies from 28% to 77%, the administration of antilymphocyte globulin (ALG) has been considered effective in treating severe aplastic anemia. Efforts have been made to find a relationship between those patients who respond to ALG therapy and the results of in vitro tests or some pretreatment clinical variable to predict those likely to respond to ALG. Since the introduction of ALG therapy for aplastic anemia, it is believed that ALG exerts its cytotoxic effect on lymphocytes in a complement-dependent manner in vivo as well as in vitro, and indirectly improves the suppressed hematopoiesis. Although several studies have attempted to clarify the mode of ALG action in this respect, the findings are not in agreement. Recent in vitro observations indicate that ALG can exert a mitogenic effect on the lymphocytes and induce them to produce hematopoietic growth factors. This mechanism for the response to ALG treatment is supported by findings in treating aplastic anemia with recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) and with rhG-CSF. Assuming that the immunostimulatory effects of ALG lead to an improvement of the bone marrow hypoplasia, we investigated the correlation between the clinical response observed with ALG therapy of aplastic anemia and the lymphocyte stimulatory effect of this agent in vitro.

MATERIALS AND METHODS

Patients. Eighteen patients with aplastic anemia, 10 males and eight females aged 2 to 43 years, were included in the study. Their clinical and laboratory data are summarized in Table 1. Of the 18 patients, 14 had severe aplasia according to the criteria of the International Cooperative Aplastic Anemia Study Group. The cause of the aplasia was acute hepatitis in one patient, but etiology was unknown in 17. The duration between verification of the diagnosis to the initiation of therapy ranged from 2 weeks to 52 months. Nine patients had acute disease (ie, diagnosed within 3 months of initiating the study), while nine had chronic disease. This study was approved by the Review Board of the Nagoya University School of Medicine. Patients were informed that blood samples were obtained for research purposes and that their privacy will be protected.
cin (RPMI-FCS). PBMCs from six patients were cryopreserved until tested under the same conditions as with the assay systems.

**Proliferative response of PBMCs.** To assess the proliferative capability of the patients' PBMCs, the cells were cultured in RPMI-FCS at 1 x 10^6 cells per 200 μL in triplicate using U-bottom 96-well plates (Nunc, Roskilde, Denmark) with various concentrations of ALG (lot 113GU) or horse serum IgG (lot 28839; Cappel, West Chester, PA). Plates were settled in a humidified atmosphere containing 5% CO2 in air at 37°C for 70 hours. Cells were subsequently pulsed for 2 hours with 18.5 kBq of 3H-thymidine and harvested. Radioactivity was determined in a liquid scintillation counter.16 Because of the variability of the biologic assay, PBMCs were also cultured with 2.5 μg/200 μL phytohemagglutinin (PHA-P; Difco, Detroit, MI) under the same conditions as in the case of ALG. The FCS, ALG, and horse serum IgG used in these studies were heat-inactivated at 56°C for 30 minutes.

**GM-CSF assay.** Supernatants for GM-CSF assay were prepared as follows: peripheral blood mononuclear cells from 14 of 18 patients, including 7 of 8 responders and 7 of 10 nonresponders, were cultured in RPMI-FCS with ALG (250 μg/mL) or PHA (10 μg/mL) in a culture tube (Falcon 3033; Becton Dickinson, Lincoln Park, NJ) at 1 x 10^6 cells/mL for 48 hours. Cells were subsequently pelletted and the supernatants recovered and stored at −80°C until studied. GM-CSF concentrations in each culture supernatant were determined by enzyme-linked immunosorbent assay (ELISA; Enzyme, Boston, MA). The lowest level of GM-CSF detectable with this ELISA system was 0.001 ng/mL. The spontaneous production of GM-CSF from the patients' PBMCs were tested in parallel.

**Two-color flow cytometry analysis of PBMCs cultured with ALG.** Surface analysis of the PBMCs, cultured with ALG for 48 hours, was conducted by direct immunofluorescence using monoclonal antibodies (MoAbs). The latter were fluorescein isocyanate (FITC)-conjugated anti-CD3, anti-CD4, and anti-CD8 MoAbs (Coulter Immunology, Hialeah, FL), and phycoerythrin (PE)-conjugated anti-CD25 MoAb (DakoCytomation, Glostrup, Denmark). Cultured cells were incubated with anti-CD25 MoAb and one of the anti-T-cell MoAbs simultaneously at 4°C for 30 minutes. Flow cytometry analysis was performed using a fluorescence-activated cell sorter (EPICS PROFILES; Coulter).

**RESULTS**

**Clinical response.** Of the 18 patients, eight (44%) showed a response to ALG therapy and 10 (56%) had no response 3 months after the initiation of treatment. Of the eight responders, six were maintained in remission at 12 months and two relapsed at 12 and 36 months after the initial response, one of whom responded to a second course of ALG therapy. There was no significant difference between responders versus nonresponders as to their age, sex, severity of disease, duration to the initiation of therapy, pretreatment peripheral blood counts (granulocytes, reticulocytes, or platelets), or pretreatment blood transfusions.

The severity of aplastic anemia was assessed according to the criteria of the International Cooperative Aplastic Anemia Study Group.16 A total of eight patients (nos. 1 through 8) were responders, while 10 patients (nos. 9 through 18) were nonresponders. There was no significant difference between responders v nonresponders (P > .1) with respect to age, sex, severity of disease, duration to the initiation of therapy, pretreatment peripheral blood counts (granulocytes, reticulocytes, or platelets), or pretreatment blood transfusions.

**Table 1. Patient Characteristics**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Etiology of Aplastic Anemia</th>
<th>Duration of Disease (mo)</th>
<th>Severity of Aplastic Anemia*</th>
<th>Pretreatment Peripheral Blood Counts (x 10^9/L)</th>
<th>Blood Transfusions (U)</th>
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<td></td>
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<td></td>
<td></td>
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<td>0.48</td>
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<tr>
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<td>32</td>
<td>Severe</td>
<td>1.0</td>
<td>1.0</td>
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</tbody>
</table>

A total of eight patients (nos. 1 through 8) were responders, while 10 patients (nos. 9 through 18) were nonresponders. There was no significant difference between responders v nonresponders (P > .1) with respect to age, sex, severity of disease, duration to the initiation of therapy, pretreatment peripheral blood counts (granulocytes, reticulocytes, or platelets), or pretreatment blood transfusions.

*The severity of aplastic anemia was assessed according to the criteria of the International Cooperative Aplastic Anemia Study Group.16
ALG'S LYMPHOSTIMULATORY EFFECT IN AA THERAPY

Fig 1. Dose-response curves of PBMCs from 18 patients with aplastic anemia to ALG (---) and horse IgG (----). PBMCs were cultured with ALG or horse IgG for 72 hours. Proliferation of cells was subsequently assessed by H-thymidine incorporation. Results are expressed as \( \text{Im} \pm \text{SD (cpm)} \) from each group of patients (responder, [O]; nonresponder, [O]). Results obtained with these groups differed significantly \((P < .01)\) for ALG (500 \( \mu \)g/mL) when compared by two-way analysis of variance. No statistical difference for horse IgG was found between nonresponders versus responders \((P > .1)\).

Data from the responders and nonresponders are presented in Fig 2. The responders (logarithmic mean \([\text{Im}] = 11,111, \text{Im} + \text{SD} = 16,710, \text{Im} - \text{SD} = 7,387 \text{cpm}\)) differed significantly from the nonresponders \((\text{Im} = 4,229, \text{Im} + \text{SD} = 9,356, \text{Im} - \text{SD} = 1,912 \text{cpm}\)) for ALG (500 \( \mu \)g/mL) according to two-way analysis of variance \((P < .01)\). No significant difference for PHA-P or horse IgG was found between the responders and nonresponders.

GM-CSF assay in culture supernatants from PBMCs stimulated with ALG. The distribution of GM-CSF produced by the ALG-stimulated PBMCs is shown in Fig 3C. The results obtained with the responders \((\text{Im} = 0.0293, \text{Im} + \text{SD} = 0.1258, \text{Im} - \text{SD} = 0.0068 \text{ng/mL}\)) differed significantly from the nonresponders \((\text{Im} = 0.0014, \text{Im} + \text{SD} = 0.0025, \text{Im} - \text{SD} = 0.0008 \text{ng/mL}; P < .01)\). The production of GM-CSF by the PBMCs of all but one responder ranged from 0.012 to 0.3 ng/mL. The single exception was the patient with hepatitis-associated aplastic anemia in whom the ALG-stimulated PBMCs could produce GM-CSF as 0.005 ng/mL among the responders. The concentration of GM-CSF in the supernatant from the PBMCs of five of the seven nonresponders was below 0.001 ng/mL, the lowest limit detected by this assay system. No significant difference for PHA-P was found between the responders and nonresponders.

Surface phenotype of PBMCs stimulated with ALG. An activated subpopulation of T lymphocytes in the PBMCs from three responders was analyzed with anti-CD25 MoAb using two-color flow cytometry after the incubation of PBMCs with ALG for 48 hours. A representative result appears in Fig 4. Before treatment, the expression of CD4, CD8, and CD25 in these patients' fresh PBMCs was examined; the means \( \pm \text{SD} \) were 43.1% \( \pm \) 5.3%, 41.3% \( \pm \) 7.6%, and 0.7% \( \pm \) 0.3%, respectively. Following incubation of PBMCs with ALG, the CD25 antigen was expressed for 13.3% \( \pm \) 7.5% of the CD3-positive cells, predominantly among the CD4-positive rather than the CD8-positive T cells. The CD4-positive subpopulation was 78.8% \( \pm \) 8.5%, whereas the CD8-positive cells were 21.0% \( \pm \) 7.3% of the CD25-positive cells. Nonimmune horse IgG induced little CD25 antigen expression \((<1.9\% \text{ of the CD3-positive cells})\).

DISCUSSION

Because immunocompetent cells are involved in the pathogenesis of aplastic anemia,\(^{16}\) ALG is believed to restore the depressed hematopoiesis through a cytotoxic action on such cells. Because it has been difficult to demonstrate that the effectiveness of ALG in treating aplastic anemia is due to its cytotoxicity, an alternative...
Fig 3. Distribution of GM-CSF production by PBMCs from 12 patients with aplastic anemia cultured in RPMI-FCS (A), with PHA stimulation (B), or ALG (C). The four patients consisted of one responder and three nonresponders (nos. 6, 9, 14, and 16), and were excluded from the GM-CSF assay because of the marked lymphocytopenia and the difficulty preparing the adequate number of PBMCs in these cases. Each dot represents one patient (responder, [ ]; nonresponder, [O]). Im ± SD (nanograms per milliliter) of GM-CSF concentration in culture supernatants are also shown. Results obtained in the responders and nonresponders differed significantly (P < .01) when PBMCs were cultured with ALG (C) (responders: Im = 0.0293, Im ± SD = 0.1258, Im - SD = 0.0068 ng/mL; nonresponders: Im = 0.0014, Im ± SD = 0.0025, Im - SD = 0.0008 ng/mL). (*), Patient with hepatitis-associated aplastic anemia; (t), moderate cases.

explanation is required. Since the lymphostimulatory potencies of ALG on the PBMCs from normal controls10,12 and from patients with aplastic anemia13-15 have been identified, the extent of the effectiveness of ALG that can be attributed to its lymphostimulatory potency remains to be determined. Previously, Kawano et al14 reported a difference of lymphostimulatory activity among ALG preparations, speculating that this could explain in part the variation in its clinical effectiveness. In this study, we evaluated the clinical relevance of the lymphostimulatory potency of ALG and showed a correlation between the clinical response to ALG therapy and its in vitro lymphocyte stimulatory effects. The clinical response of the patient was correlated with the proliferative response and the extent of the GM-CSF production by ALG-stimulated PBMCs.

The subpopulations that would proliferate were suggested by two-color flow cytometry analysis of CD25-positive cells of PBMCs stimulated by ALG. Endogenous interleukin-2 (IL-2) is considered to be one of the major cytokines that induces the proliferation of lymphocytes and is a natural ligand of CD25 antigen. Therefore, the cells that predominantly proliferate in short-term culture would be expected to be CD4-positive rather than CD8-positive. In a study at the clonal level, ALG stimulated both of the CD4- and CD8-positive subsets, but the CD4 antigen was predominantly expressed among the ALG-stimulated blasts in contrast to PHA-stimulated blasts. This does not inevitably imply that only the CD4-positive subset will release various hematopoietic factors when incubated with ALG. At least in long-term culture, both CD8- as well as CD4-positive cells would produce GM-CSF.12

Investigation of the cytokines known to exert hematopoietic activities when secreted by ALG-stimulated PBMCs would help to explain the process from the proliferation of PBMCs induced by ALG until the enhancement of hematopoiesis. We demonstrated a significant difference of ALG-induced GM-CSF production of PBMCs between the responders and the nonresponders, and suggested that ALG could restore the depressed hematopoiesis by an upregulation of the GM-CSF production of the patients'
PBMCs. Other hematopoietic factors such as G-CSF and IL-3 would also be expected to be produced by PBMCs stimulated with ALG.

Our discussion of the lymphocyte stimulatory potencies of ALG does not necessarily disagree with previous reports that suggest the clinical importance of immunosuppressive agents. One such report is the downregulation of γ-interferon production induced by ALG in the PHA-stimulated patients' PBMCs and the correlation of clinical response to ALG with this activity of ALG. The recently reported excellent results with cyclosporine in aplastic anemia seem to suggest that an immunosuppressive mode of action of the therapeutic agent is essential, in comparison to the transient improvement obtained with GM-CSF administration.

Thus, the possibility is far from slight that ALG primarily exerts an immunosuppressive effect and that residual hematopoietic potency is essential for a response to ALG. However, our results with ALG therapy did not show a correlation between the response to ALG and the severity of disease. In addition, in a previous study we failed to detect the suppressor T cells to hematopoietic progenitors by comparing the growth of GM colony-forming cells and burst-forming units before and after treating bone marrow cells with anti-T-cell MoAbs and complement in patients with aplastic anemia. The clinical significance of the direct cytotoxicity of ALG to lymphocytes, especially to the T lymphocytes expected to be suppressive to hematopoiesis, is still controversial. It would be necessary to examine further the immunosuppressive potency of ALG on other kinds of cells, including various subsets of lymphocytes, monocytes, and stroma cells.

Considering the pathophysiology of aplastic anemia and the mode of action of ALG, a possible therapeutic strategy would be to administer a hematopoietic factor such as GM-CSF, particularly if the clinical response is positively correlated with the in vitro production of the patients' PBMCs induced by ALG, given in combination with immunosuppressive therapy.

Antilymphocyte globulin has two different effects on the cells that are committed to the immune system, immunostimulatory and immunosuppressive, apparently in opposition to each other, but which, acting together, restore the suppressed hematopoiesis in some patients with aplastic anemia. Our results indicate that the clinical response to ALG is correlated with the lymphocyte proliferative response induced by this agent in vitro, and suggests the utility of an in vitro test for predicting the clinical response to ALG therapy.

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