RAPID COMMUNICATION

Interferon-γ Gene Expression in Unstimulated Bone Marrow Mononuclear Cells Predicts a Good Response to Cyclosporine Therapy in Aplastic Anemia

By Shinji Nakao, Masaki Yamaguchi, Shintaro Shiobara, Toru Yokoi, Toshio Miyawaki, Takashi Taniguchi, and Tamotsu Matsuda

Cyclosporine (CyA) therapy has been shown to be effective for some patients with aplastic anemia (AA).1,2 It would be extremely valuable to be able to predict those patients who would respond to CyA therapy particularly as alternative treatments such as bone marrow (BM) transplantation are associated with significant risks. A good way of predicting a favorable response, however, has not been found as yet. Previous studies have shown abnormal lymphokine production in patients with AA and related these abnormalities to hematopoietic stem cell suppression.3,4 Because the main action of CyA is considered to be inhibition of cytokine production by T lymphocytes,5 the improvement in AA induced by CyA suggests that lymphokine overproduction could be important in the pathogenesis of AA and this may be corrected by the administration of CyA. On the basis of this hypothesis, we studied BM cells from patients with AA who were then treated with CyA. Gene expression of several lymphokines was examined, all of which are known to have inhibitory effects on in vitro growth of hematopoietic progenitor cells.

MATERIALS AND METHODS

Patients. Twenty-three patients with AA (severe 16, moderate 7) were treated with oral CyA alone for at least 3 months. Eighteen of these patients were administered high-dose methylprednisolone (HDMP) without appreciable effects before CyA therapy, but only four of them had been treated with antithymocyte globulin (ALG). All patients except no. 4 were transfusion-dependent for red blood cells at initiation of CyA therapy. The response criteria were resolution of all transfusion requirements and improvement of hemoglobin levels above 8 g/dL.

Preparation of BM cells. Heparinized BM (0.5 to 1.0 mL) was collected from the 23 aplastic patients before CyA therapy, 10 normal volunteers, five patients with myelodysplasia, and four patients with acute leukemia in remission after giving informed consent. For the seven aplastic patients who responded to CyA therapy, another BM was obtained after hematologic improvement had occurred. BM mononuclear cells (BMMC) were isolated by Ficoll-Hypaque density gradient centrifugation. A total of 10^6 BMMC were immediately homogenized with 0.5 mL of the denaturation solution containing guanidium thiocyanate.6 Aliquots of BMMC in the denaturation solution were stored in a deep freezer at −80°C until use for RNA extraction.

cDNA synthesis and polymerase chain reaction (PCR). Total RNA was extracted from 10^6 BMMC in the denaturation solution using the guanidine-phenol method.6 For PCR, cDNA was synthesized from the RNA in 50 μL of reaction mixtures by using RAV-2 reverse transcriptase (Takara, Kyoto, Japan) with oligo dT as a primer. Synthesized cDNA was purified with a sephadex G-50 spin column. One microliter of the final cDNA preparation was added to 24 μL of each reaction mixture containing 1.0 U of Taq polymerase for the amplification of cDNA for tumor necrosis factor (TNF),7 macrophage inflammatory protein-1α (MIP-1α),8 lymphokinin, interferon-γ (IFN-γ),9 and β-actin.7 The specific primers used for amplification of each cytokine and size of amplified fragments are shown in Table 1. After 30 cycles consisting of 1 minute of denaturation at 94°C, 2 minutes of annealing at 55°C, and 2 minutes of primer extension at 72°C, 10 μL of the PCR product was electrophoresed in a 2% agarose gel and was examined for the presence of a band of the expected size after ethidium bromide staining. PCR-assisted messenger RNA (mRNA) amplification was repeated at least once for each sample. A cDNA preparation made from phytohemagglutinin-stimulated lymphocytes and a water blank were included in every PCR. When separate experiments gave conflicting results, RNA was extracted from another aliquot of BMMC in the denaturation solution and the following procedure for PCR amplification was repeated using new aliquots of buffers, primers, and enzymes.

In an attempt to characterize aplastic patients likely to benefit from CyA therapy, we examined bone marrow mononuclear cells (BMMC) obtained before therapy from 23 patients with aplastic anemia, who were treated with CyA alone. Expression of four myelosuppressive cytokines, including tumor necrosis factor (TNF), lymphokinin, macrophage inflammatory protein-1α (MIP-1α), and interferon-γ (IFN-γ) was examined using polymerase chain reaction (PCR)-assisted messenger RNA (mRNA) amplification. mRNA for TNF, lymphokinin, and MIP-1α was readily detectable at variable levels in BMMC from normal and transfused controls as well as in BMMC from aplastic patients. In contrast, IFN-γ mRNA was only demonstrable in BM from some patients with aplastic anemia, irrespective of a history of transfusions. Of 13 patients who responded to CyA therapy and achieved transfusion-independence, IFN-γ mRNA was detected in 12 patients, whereas the mRNA was only detectable in 3 of 10 patients refractory to CyA therapy (P = .003, Fisher’s exact test). Follow-up examination of BMMC obtained from seven CyA-responding patients after hematologic remission showed disappearance of IFN-γ mRNA in four patients. These results suggest that detection of IFN-γ gene expression in pretreatment BMMC from aplastic patients using PCR may be helpful in predicting a good response to CyA therapy.

© 1992 by The American Society of Hematology.

From the Third Department of Medicine, Department of Pediatrics, Kanazawa University School of Medicine, Kanazawa, Japan.

Submitted January 10, 1992; accepted March 13, 1992.

Address reprint requests to Shinji Nakao, MD, Third Department of Medicine, Kanazawa University School of Medicine, 1-1 Takaramachi, Kanazawa, Ishikawa 920, Japan.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1992 by The American Society of Hematology.

0006-4971/92/7910-0046$3.00/0

Table 1. Primer Sequences Used for PCR-Assisted mRNA Amplification

<table>
<thead>
<tr>
<th>mRNA</th>
<th>5’ Sense Primer</th>
<th>3’ Antisense Primer (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF</td>
<td>ATGAGCAGCAAGAG-</td>
<td>TCACAGGGCAAT-TGATGCTC</td>
</tr>
<tr>
<td>Lymphotoxin</td>
<td>GTGACCCCACT-</td>
<td>GATGCCAAAAGTA-GAAAATG</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>GTGACCTGTCCAC-</td>
<td>TGCTG-TGTCGGCTGCAG</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>GGCTTTTCAGCTCT-</td>
<td>GATGCTCTCTCA-GCCATGCTG</td>
</tr>
<tr>
<td>β-actin</td>
<td>GTGGGGGCGCCCGAC-</td>
<td>GCACCA-CACGCAGAATTCCAGAT-</td>
</tr>
</tbody>
</table>

RESULTS

Figure 1 shows results of PCR analysis for TNF, lymphotoxin, MIP-1α, IFN-γ, and β-actin genes on 16 subjects, including normal individuals, transfused controls, and aplastic patients who responded or did not respond to CyA therapy. Gene expression of TNF, lymphotoxin, and MIP-1α was detectable at varying degrees in BMMC from all four different groups, although it appeared to be increased in BMMC from aplastic patients who responded to CyA therapy. In contrast, IFN-γ gene expression was only detected in BMMC from some patients with AA, irrespective of a history of blood transfusions (Table 2). IFN-γ mRNA was detected in 12 of 13 CyA-responding patients, whereas in patients refractory to CyA therapy, IFN-γ mRNA was detected in only 3 of 10 patients ($P = .003$, Fisher’s exact test). There was no relationship of IFN-γ mRNA to prior transfusions ($P = .069$), prior immunosuppressive therapy ($P = .36$), and age ($P = .24$, Student’s $t$-test). IFN-γ gene expression was undetectable in four of seven CyA-responding patients when their BMMC were examined after they had become transfusion-independent. Because sufficient number of BMMC were available for the four CyA-responding patients (nos. 1, 3, 5, and 8) who were positive for IFN-γ gene expression before therapy,
surface phenotype of T lymphocytes in the BMMC and spontaneous IFN-γ production from the BMMC were determined. Proportions of CD4⁺ and CD8⁺ cells (%CD4/%CD8) among BMMC from these four patients were 5.9/5.8, 14.4/14.7, 12.3/13.5, and 4.4/4.6, respectively. These values were comparable with those of controls (%CD4/%CD8) among BMMC from normal individuals. In contrast, proportions of HLA-DR⁺ cells among CD8⁺ cells were markedly high in every patient at 72%, 61%, 53%, and 61% compared with normal controls (32% ± 8%). Spontaneous IFN-γ production from BMMC were shown in one patient (no. 5, 3.5 IU/mL), but not in the other three patients.

**DISCUSSION**

Our studies showed IFN-γ gene expression in unstimulated BMMC from some patients with AA, particularly those who responded to CyA therapy. Expression of genes for the other myelosuppressive factors, including TNF, lymphotoxin, and MIP-1α, was detected nonspecifically in BMMC from normal individuals and transfused controls. Few studies have focused on expression of marrow suppressive cytokine genes in unstimulated BM cells. Ehlers and Smith showed that mRNA for cytokines, including IFN-γ and TNF, was not detectable in unstimulated peripheral blood mononuclear cells (PBMC) using PCR-assisted mRNA amplification. In our hands, mRNA of TNF and MIP-1α were readily detectable in PBMC from normal individuals in addition to BMMC, although lymphotixin gene expression was confined to BMMC from only some of them (data not shown). The discrepancy between this study and Ehlers and Smith’s study may be due to different primers used to amplify cDNA for each cytokine. Because both TNF and MIP-1α are known to be mainly produced by monocytes, the mRNA detected in unstimulated mononuclear cells may be due to physical stimulation of monocytes associated with mononuclear cell isolation.

Overproduction of myelosuppressive lymphokines in patients with AA has been documented by several reports. Zoumbos et al suggested that IFN-γ was a mediator of hematopoietic suppression in AA. Hinterberger et al confirmed their results, although others were unable to show overproduction of IFN-γ in AA. Although spontaneous IFN-γ production from BMMC was shown in one of four patients whose BMMC were shown to express IFN-γ gene in the present study, it is possible that low levels of IFN-γ were overproduced in the BM microenvironment and this directly inhibited hematopoietic stem cell growth in vivo; this might be expected given that the local concentration of IFN-γ in the marrow would determine progenitor cell suppression. This suppression might be then removed through suppressive effects of CyA on IFN-γ production by T lymphocytes.

Alternatively, the IFN-γ gene expression in unstimulated BMMC may be related to ongoing specific immune-reactions involving hematopoietic progenitor cells. The role of IFN-γ in the pathogenesis of several autoimmune disorders, including insulin-dependent diabetes mellitus and multiple sclerosis, have been postulated. In these diseases, IFN-γ produced locally by T lymphocytes is consid-
ered to act on target tissue to induce HLA-DR and thereby enhance recognition of the target by CD4+ T lymphocytes, leading to the cytotoxic T-cell-mediated tissue destruction. Similar mechanisms may act on unknown antigens on hematopoietic stem cells in AA. The increased proportion of activated (HLA-DR+) CD8+ cells among total CD8+ cells in BMMC from the CyA-responding patients and the close association of HLA-DR2 with a likely good response of AA to immunosuppressive therapy support this hypothesis. CyA may act on T cells to reduce production of IFN-γ and thus reverse the events leading to exhaustion of hematopoietic stem cells.

The role of IFN-γ gene expression shown in the present study in the pathogenesis of AA remains uncertain because the gene expression was still detectable in some patients in remission after treatment with CyA. However, the results of our study indicate that detecting IFN-γ gene expression in unstimulated BMMC from aplastic patients before therapy by means of PCR-assisted mRNA amplification could be important in predicting a good response to CyA. Laver et al. showed that production of IFN-γ by cultured T cells from patients with AA correlated with response to antithymocyte globulin. Their method may be applicable to identification of potential responders to CyA as well. However, it seems extremely difficult to culture patient’s lymphocytes obtained at different times in an identical condition for precise measurement of IFN-γ production. PCR-assisted IFN-γ mRNA amplification is simple and artifacts can be avoided if appropriate positive and negative controls are examined together with test samples. Studies on a larger series of cases will further clarify the role of IFN-γ gene expression both in predicting a response to CyA and in the pathogenesis of AA.

REFERENCES

Interferon-gamma gene expression in unstimulated bone marrow mononuclear cells predicts a good response to cyclosporine therapy in aplastic anemia

S Nakao, M Yamaguchi, S Shiobara, T Yokoi, T Miyawaki, T Taniguchi and T Matsuda