

## Oligoclonal T cell expansion in blood but not in the thymus from a patient with thymoma-associated pure red cell aplasia

**Despite the well-known association between thymoma and PRCA, the role of thymoma remains uncertain. There is accumulating evidence that clonal T cells are involved in acquired PRCA. We examined T cell receptor repertoires in blood and thymus from a patient with PRCA associated with thymoma and myasthenia gravis. Oligoclonal expansions of V $\beta$ 1- and V $\beta$ 1-expressing T cells were found in peripheral blood, whereas the repertoires of V $\beta$ 1+ and V $\beta$ 1+ T cells in thymoma were not skewed. Oligoclonal expansion of V $\beta$ 1-expressing T cells remained unchanged after thymectomy. Thymus may not be the site of clonal T cell expansion in thymoma-associated PRCA.**

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Pure red cell aplasia (PRCA) is a syndrome characterized by selective inhibition of hematopoiesis for the erythroid lineage.<sup>1</sup> It is generally believed that acquired PRCA is principally mediated by the autoimmune mechanism. There are several reports demonstrating the presence of clonal T cell expansions in PRCA.<sup>2,3,4</sup> Handgretinger *et al.* clearly demonstrated the association of PRCA with clonal expansion of granular lymphocytes expressing the V $\beta$ 1 T cell receptor (TCR).<sup>5</sup> They suggested that the killer-cell inhibitory receptors are involved in cytotoxicity against erythroid progenitors because of their low expression of HLA class I molecules. Therefore, it is reasonable to assume that clonally expanded T cells are involved in the inhibition of erythropoiesis in acquired PRCA patients, although the antigen specificity of these T cell clones is largely unknown.

Secondary acquired PRCA is complicated by various disorders including thymoma, hematologic malignancies, solid tumors, infections, or collagen diseases. However, the role of thymus in the pathogenesis of thymoma-associated PRCA remains uncertain, since some patients develop PRCA after thymectomy or treatment of thymoma by radiation or chemotherapy.<sup>6</sup> We recently encountered a patient with thymoma-associated myasthenia gravis followed by the development of PRCA, in whom we were able to examine the clonality of T cells in blood and thymic tissues. We found oligoclonal T cell expansion in the blood but not in the thymus.

### Case report

A 52-year-old Japanese woman was diagnosed as having myasthenia gravis associated with thymoma in 1990. At the onset of myasthenia gravis, a hemoglobin level was 11.0 g per deciliter with a red cell count of 4.36 million per cubic millimeter, hematocrit of 33.2 percent, reticulocytes of 3.52 percent (0.153 million per cubic millimeter), a platelet count of 389,000 per cubic millimeter, and a white cell count of 7000 per cubic millimeter with 53 percent neutrophils and 29 percent lymphocytes. She required the mechanical ventilation support for severe respiratory muscle paralysis. Her critical condition responded to high dose steroid followed by oral prednisolone. She refused thymectomy at that time. Remaining blepharoptosis disappeared after additional daily 2 mg oral tacrolimus. She developed severe anemia in October 2004, and was referred to our department.

The hemoglobin level was 4.4 g per deciliter, with a red

cell count of 1.15 million per cubic millimeter, hematocrit of 13.0 percent, and 0.28 percent (3200 per cubic millimeter) reticulocytes. The platelet count was 363,000 per cubic millimeter and the white cell count was 4000 per cubic millimeter with 3480 per cubic millimeter neutrophils, 360 per cubic millimeter lymphocytes, 160 per cubic millimeter monocytes. Direct and indirect anti-Coombs tests were negative. Renal and liver function tests were normal. The serum anti-acetylcholine antibody level was 145 nmol per liter (normal range; <0.2). Anti-human parvovirus B19 IgM was not detected by enzyme-immunoassay. Bone marrow was normocellular with 1.0 percent erythroblasts, without giant proerythroblasts, and the myeloid to erythroid ratio was 53.7. There was no proliferation of myeloblasts. Cytogenetic analysis of bone marrow cells showed a normal karyotype. Chest computed tomography demonstrated the presence of thymoma without invasion to surrounding tissues, and no remarkable change in the size as compared with those previously taken. She did not have a previous history of taking drugs that might cause PRCA such as anticonvulsants, antibiotics, anti-viral agents, or viral infections preceding this episode. Hence, she was given a diagnosis of thymoma-associated PRCA with preceding myasthenia gravis. She required regular red cell transfusion. After obtaining informed consent, thymectomy was performed. Histological diagnosis was type B2 thymoma according to the WHO classification. She was still dependent on red cell transfusion for a while even after thymectomy. Blood trough levels of tacrolimus were approximately 3 ng per milliliter on taking 2 mg each day. Three months after thymectomy, she was given cyclosporine at a dose of 250 mg per day in place of tacrolimus, when blood trough levels were approximately 180 ng per milliliter. Reticulocyte counts increased, and she became free of transfusion. Hemoglobin levels normalized up to 12.3 g per deciliter with 58300 per cubic millimeter reticulocytes seven months after starting cyclosporine.

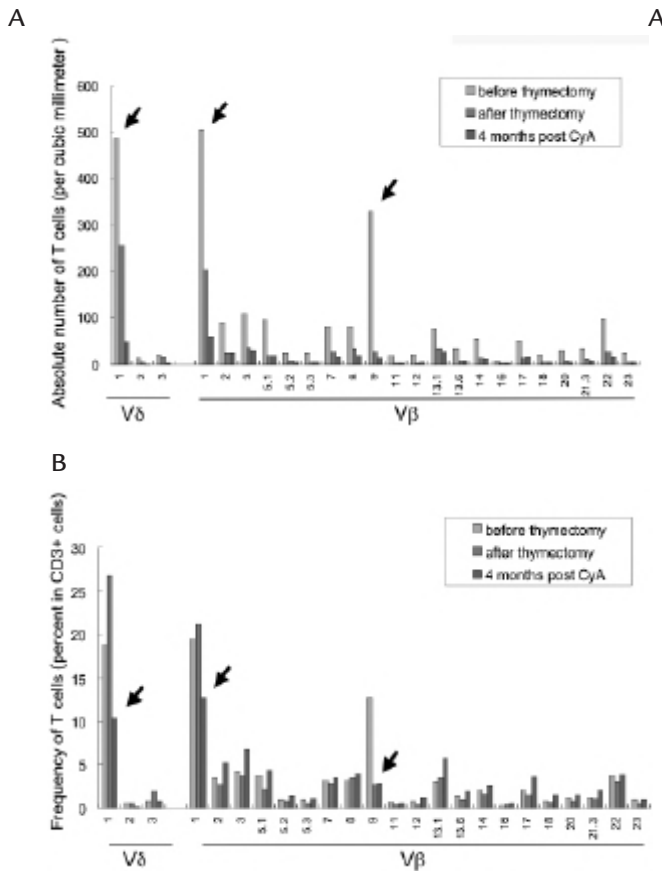
### Material and methods

#### Flow cytometry

The experimental protocol was approved by the institutional review board and the written informed consent was obtained from the patient. The monoclonal antibodies used in this study were as follows: anti-CD3 (Becton Dickinson, San Jose, CA, USA); anti-TCR- $\beta$ -1 (Becton Dickinson, San Jose, CA, USA); anti-V $\beta$  subfamilies (V $\beta$ 1, V $\beta$ 2, V $\beta$ 3; Immunotech, Marseille, France); anti-V $\beta$  subfamilies (Immunotech, Marseille, France) and control mouse IgG (eBioscience, San Diego, CA, USA). Stained cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA).

#### CDR3 size spectratyping of TCRDV and TCRBV

The size of complementarity-determining region 3 (CDR3) of TCR  $\beta$ - and  $\delta$ -chains was determined as described elsewhere.<sup>7,8</sup> Total RNA was extracted from freshly isolated PBMCs and thymic tissues using an RNeasy Total RNA Kit (Qiagen, Hilden, Germany), and was used for first-strand cDNA synthesis with an oligo-dT primer (First-Strand cDNA Synthesis Kit, Amersham, Uppsala, Sweden). Aliquots of the cDNA were amplified with the V $\beta$ -specific oligonucleotide and the C $\beta$  primer for CDR3 size spectratyping of TCR  $\beta$  chains, and with the V $\delta$ -specific oligonucleotide and the C $\delta$  primer for TCR  $\delta$  chain analysis. The sequences of primers have been described previously.<sup>7-10</sup> The unlabeled PCR products were subjected to one cycle of elongation with a FAM-



**Figure 1.** TCRBV and TCRDV repertoires before and after thymectomy and CyA therapy. T cell repertoires were analyzed by flow cytometry. V $\beta$ 1+ $\beta$ 7T cell, V $\beta$ 1+ and V $\beta$ 9+  $\beta$ 7T (arrows) in peripheral blood were expanded more than that in normal individuals at the onset of PRCA. Frequencies of V $\beta$ 1-, V $\beta$ 1- and V $\beta$ 9- expressing peripheral blood T cells in healthy individuals were  $1.2 \pm 0.5$  percent ( $19 \pm 14$  per cubic millimeter),  $3.9 \pm 1.0$  percent ( $64 \pm 35$  per cubic millimeter), and  $3.4 \pm 1.1$  percent ( $51 \pm 6$  per cubic millimeter) (mean  $\pm$  SD of 3 samples), respectively. The first flow cytometric analysis of TCR repertoires was performed one month after the onset of PRCA.

labeled C $\gamma$  or C $\delta$  primer The labeled PCR products were electrophoresed on acrylamide sequencing gels in an automated DNA sequencer (ABI 377, Perkin-Elmer Applied Biosystems, Foster, CA, USA), followed by analysis using GeneScan software (Perkin-Elmer)

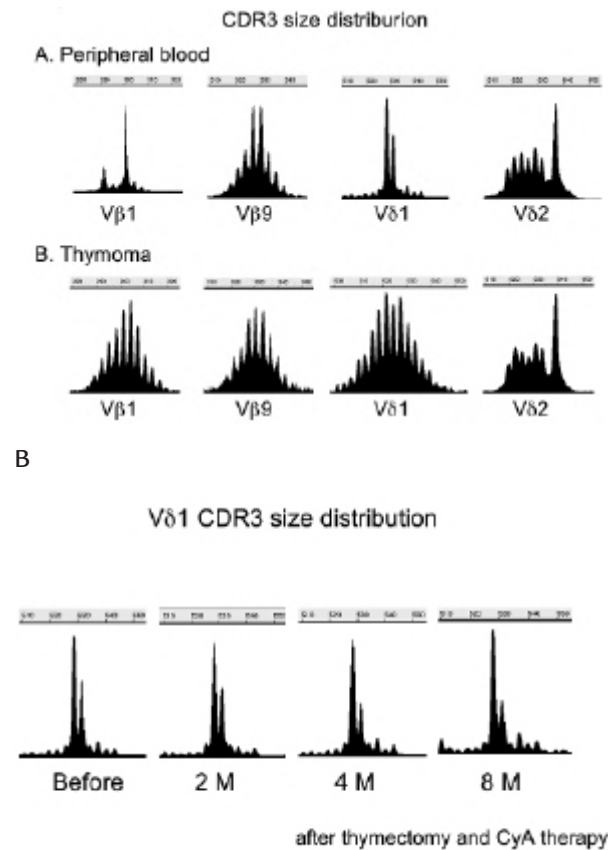
*Sequencing of CDR3 regions*

PCR products were cloned into the PCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA) and were sequenced using a Big-Dye Terminator Cycle Sequencing Kit (Perkin-Elmer) Sequence analysis was performed using an automated DNA sequencer.

**Results**

*TCRBV and TCRDV repertoires of T lymphocytes in blood and thymoma*

We first examined the usage of TCRBV and TCRDV subfamilies by peripheral blood T lymphocytes to determine whether there was a skew of TCR variable region repertoires As compared to those of healthy individuals, the usages of V $\beta$ 1, V $\beta$ 1 and V $\beta$ 9 segments were increased (Figure 1A and 1B)<sup>11,12,13</sup> V $\beta$ 1+  $\beta$ 7T cells accounted for 18.8



**Figure 2.** CDR3 size distribution patterns of T lymphocytes in peripheral blood and thymoma. V $\beta$ 1- and V $\beta$ 1- expressing T cells were skewed in blood but not in the thymoma (2A). Skewed pattern of the V $\beta$ 1 TCR repertoire were persisting even after successful treatment with thymectomy and cyclosporine (2B).

percent of the CD3 $^+$  T cell population with an absolute number of 487 per cubic millimeter Whereas V $\beta$ 2+  $\beta$ 7T cells, that are usually the major subtype of circulating  $\beta$ 7T cells, accounted for only 0.5 percent with an absolute number of 15 per cubic millimeter V $\beta$ 1+ and V $\beta$ 9+  $\beta$ 7T cells accounted for 19.6 percent (506 per cubic millimeter), and 12.8 percent (332 per cubic millimeter) respectively CDR3 size distribution analysis demonstrated that V $\beta$ 1+ and V $\beta$ 1+ T cells were comprised of oligoclonally expanded clones (Figure 2A) In contrast, the diversity of V $\beta$ 1+ and V $\beta$ 1+ T cells in the thymus were polyclonal Oligoclonal expansion of T cells in blood but not in the thymoma was also confirmed by sequencing analysis of the CDR3 region of TCR  $\alpha$ - and  $\beta$ -chains (Table 1).

*Longitudinal analysis of TCR repertoires after thymectomy and CyA therapy*

We studied the longitudinal kinetics of T cell repertoires before and after thymectomy and cyclosporine therapy The numbers of V $\beta$ 1+, V $\beta$ 1+ and V $\beta$ 9+ T cells, which were expanded in the present case, decreased along with treatment (Figure 1A) Although the absolute numbers of expanded V $\beta$ 1+ and V $\beta$ 1+ T cells were reduced after thymectomy and cyclosporine therapy, these subsets remained predominant in circulating blood (Figure 1B) Moreover, the skewed diversity of V $\beta$ 1+ T cells remained the same (Figure 2B), indicating that oligoclonally expanded T cells were still present during hematological remission.

**Table 1.** Oligoclonally expanded T cells in blood were not detected in the thymus.

TCR	V segment	n-D-n	J segment	Colony Frequency	
				Blood	Thymoma
TCRDV1	YFCALGE	LGPYGTGGS	YTDKLIKFGK	7/32	0/31
	YFCALGE	LPGGGYQIP	YTDKLIKFGK	3/32	0/31
	YFCALGE	LVGIPSTGG	YTDKLIKFGK	3/32	0/31
TCRBV1	LYFCASSV	GGEA	GELFFGEG	11/13	0/13

### Discussion

We presented a case of thymoma-associated PRCA with oligoclonal expansions of TCR?? and TCR?? T lymphocytes in blood but not in the thymus Mamiya *et al.* previously reported that 17 of 150 patients were complicated with thymoma, and that 10 patients developed PRCA after thymectomy or treatment of thymoma including radiation or chemotherapy.<sup>6</sup> Other groups also reported that 4 patients with myasthenia gravis developed PRCA after thymectomy.<sup>14</sup> These findings raise the question of whether thymoma is indeed involved in the pathogenesis of PRCA. To date, there are few reports describing the T cell repertoire in thymoma-associated PRCA. We found a report describing the oligoclonal T cell expansion in thymoma but not in peripheral blood in contrast to the present case.<sup>15</sup> Type B2 thymoma has been previously reported to be associated with myasthenia gravis, but PRCA is not a common complication.<sup>16</sup> Although we expected that oligoclonally expanded T cells would be also detected in the thymoma, our findings suggest that oligoclonal T cells in the present case were selected and expanded in peripheral tissues.

There are some reports suggesting the pathological roles of V?1+ ??T cells in GLPD-associated PRCA.<sup>4,5,17</sup> Hara *et al.* have also demonstrated that ??T cells mediate an inhibition of erythropoiesis in type I autoimmune polyglanular syndrome.<sup>18</sup> This case also showed the oligoclonal expansion of V?1+ ??T cells in peripheral blood. At present, we do not have direct evidence for this T cell subset in the inhibition of erythropoiesis. No obvious improvement of anemia was observed during three months after thymectomy, while a global reduction of T lymphocytes in blood was seen after thymectomy and absolute numbers of oligoclonally expanded V?1 and V?1 T cells also decreased. However, oligoclonal expansions of T cells are still present even after achieving complete hematological response. Since cyclosporine is a functional inhibitor of T cells, cyclosporine can inhibit the function of pathogenic T lymphocytes but not eradicate those cells.

It would be an interesting to address why this patient developed PRCA during immunosuppressive therapy with tacrolimus and prednisolone, then showed amelioration after taking cyclosporine. There is a report describing the patient developing PRCA on tacrolimus after liver transplantation.<sup>19</sup> However, there is also a report that tacrolimus was effective for PRCA.<sup>20</sup> In any case, it is possible that a sufficient dose of cyclosporine was given to control the disease in the present case but the dose of tacrolimus might not have been sufficient.

In summary, the present case implies that the thymus may not be the site of clonal T cell expansion in thymoma-associated PRCA, and further investigations are necessary to address this issue. Clonal expansion of T cells in the marrow and erythroid suppressive effects of autologous T cells need to be studied in the future.

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