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SHORT REPORT

Multiple autoimmune haemopoietic disorders and insidious clonal proliferation of large granular lymphocytes

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Summary. We report a patient with clonal proliferation of $CD3^+8^+TCR\alpha\beta^+$ large granular lymphocytes (LGL) presenting multiple episodes of autoimmune cytopenia, including autoimmune neutropenia, idiopathic thrombocytopenic purpura, autoimmune haemolytic anaemia, and pure red cell aplasia. Each disorder appeared separately or as a combination during an 11-year clinical course. The increase of blood $CD3^+8^+TCR\alpha\beta^+$ LGL was detected 6 years after the initial diagnosis of cytopenia, but the absolute number of LGL cells was always $<1{\cdot}0\times10^9$/l. LGL cells were of monoclonal origin and had a chromosomal abnormality. LGL cells transiently responded to cyclosporine A therapy, which was also effective on all of these autoimmune cytopenias. Accordingly, an undetectable level of proliferation of a clonal LGL population could cause various autoimmune haemopoietic disorders.

Keywords: large granular lymphocytes, idiopathic thrombocytopenic purpura, pure red cell aplasia, autoimmune haemolytic anaemia, autoimmune neutropenia.

The lymphoproliferative disease of large granular lymphocytes (LDGL) has been classified into either $CD3^+$ LDGL of T-cell origin (T-LDGL) or $CD3^-$ LDGL of natural killer cell origin (Loughran, 1993; Mizuno *et al*, 1999; Oshimi *et al*, 1993). A majority of T-LDGL is characterized by clonal proliferation of $CD8^+$ large granular lymphocytes (LGL) that exceeds 2×10^9/l in the peripheral blood (Loughran, 1993; Oshimi *et al*, 1993). In a multicentre study of 195 cases of LDGL >90% (184 cases) fulfilled this criteria; however, LGL numbers were $1–2\times10^9$/l in eight cases and $<1\times10^9$/l in the remaining three cases (Semenzato *et al*, 1997). Since the presence of clonal $CD8^+$ T cells has been suggested in normal individuals (Posnett *et al*, 1994), it is difficult to determine a distinction between the end of normality and the beginning of pathology.

T-LDGL has been known to be frequently associated with haemopoietic cytopenia including thrombocytopenia, neutropenia and anaemia (Loughran *et al*, 1985). The mechanism of cytopenia in T-LDGL has been ascribed to cytotoxic activity of the $CD8^+$ LGL to haemopoietic cells, or to B-cell dysfunction that presumably results from abnormal suppressor function of $CD8^+$ LGL. Autoantibodies to neutrophils, platelets and erythrocytes as well as rheumatoid factor and antinuclear antibodies are frequently seen in T-LDGL, and these humoral immune abnormalities sometimes cause autoimmune neutropenia, idiopathic thrombocytopenic purpura (ITP) and/or autoimmune haemolytic anaemia (AIHA) (Brinkman *et al*, 1998; Gentile *et al*, 1996; Loughran *et al*, 1985).

We report a patient with multiple episodes of autoimmune neutropenia, ITP, AIHA and pure red cell aplasia (PRCA) who did not present a detectable level of clonal proliferation of $CD3^+CD8^+$ LGL until 6 years after the initial diagnosis.

MATERIALS AND METHODS

Surface phenotyping. The following fluorescein- or phycoerythrin-conjugated monoclonal antibodies were used: CD3 (OKT3), CD4 (OKT4), CD13 (MY7), CD20 (B1) and CD34 (MY10) (Coulter Immunology, Hialeah, Fla., U.S.A.), CD5 (Leu1), CD7 (Leu9), CD8 (Leu2a), CD16 (Leu11), CD19 (Leu12), CD56 (Leu19) and anti-HLA-DR (Becton Dickinson, Mountain View, Calif., U.S.A.), and anti-TCR$\alpha\beta$ (WT-31) and

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anti-TCR$\gamma\delta$ (T Cell Diagnostics). Blood mononuclear cells were stained, and analysed with a FACScan (Becton Dickinson). Throughout the experiment, mouse IgG_1 conjugated to fluorescein or phycoerythrin was used as a negative control.

Southern blot analysis. DNAs were extracted from $CD8^+$ blood mononuclear cells purified by using CD8-conjugated immunomagnetic beads (Dynal A.S., Oslo, Norway). 10 μg of DNA was digested with restriction enzymes, according to the suppliers' recommendations. The cut DNA was separated by a 0·8% agarose gel, transferred to a nitrocellulose membrane, and hybridized to a ^{32}P-labelled cDNA. The Jβ2 fragment of the T-cell receptor β (*TCRB*) gene was used for a cDNA probe (Akashi *et al*, 1998).

In vitro haemopoietic progenitor assay. T-cell-depleted bone marrow mononuclear cells (1×10^5) from the patient and normal volunteers were cultured in methylcellulose media containing granulocyte/macrophage colony-stimulating factor (20 ng/ml; Genzyme, Cambridge, Mass.) or interleukin-3 (20 ng/ml, Genzyme), erythropoietin (2 U/ml; Genzyme) and 20% fetal calf serum (FCS). The patient's $CD2^+$ T cells or 10^4 $CD3^+8^+$ LGL were purified by using immunomagnetic beads (Dynal A.S.) (Akashi *et al*, 1998). To evaluate the effect of the patient's T cells or LGL on the growth of myeloid progenitors, the T-cell-depleted bone marrow mononuclear cells were incubated with the purified 10^4 $CD2^+$ T cells or 10^4 $CD3^+8^+$ LGL for 24 h before the methylcellulose culture (Taniguchi *et al*, 1988). Colonies such as colony-forming unit (CFU)-granulocyte/macrophage, CFU-granulocytes, CFU-macrophages, and burst-forming unit-erythroid were counted on day 14. For the methylcellulose culture of CFU-megakaryocytes, serum from a patient with aplastic anaemia was used instead of FCS.

RESULTS

A 17-year-old woman was admitted to our hospital because of recurrent bacterial infections and persistent severe neutropenia ($<0·5\times10^9$/l) in July 1985. The clinical course of the patient is shown in Fig 1. She was diagnosed with autoimmune neutropenia in July 1986 because serum anti-neutrophil IgG antibodies were detected by a method previously reported (Takahashi *et al*, 1991). Prednisolone (PSL, 20 mg/d) was effective on autoimmune neutropenia. From March 1988 to July 1988 the patient suffered from thrombocytopenia ($<20\times10^9$/l) and neutropenia ($<0·5\times10^9$/l). She was diagnosed with ITP as well as autoimmune neutropenia because of the presence of platelet-associated IgG and increased numbers of relatively immature megakaryocytes in the bone marrow. PSL (40 mg/d) was effective for both disorders. During the course of these diseases the absolute number of blood lymphocytes as well as their CD4/CD8 ratio were within normal ranges, and there was no increase in numbers of morphologically determined LGL in blood ($0·2–0·26\times10^9$/l). Clonal rearrangement of *TCRB* gene was undetectable in blood mononuclear cells.

On July 1991 the patient presented with severe anaemia and thrombocytopenia. The diagnosis of PRCA was made because the Coombs test was negative, all erythroid components in the bone marrow were severely suppressed (2·8%

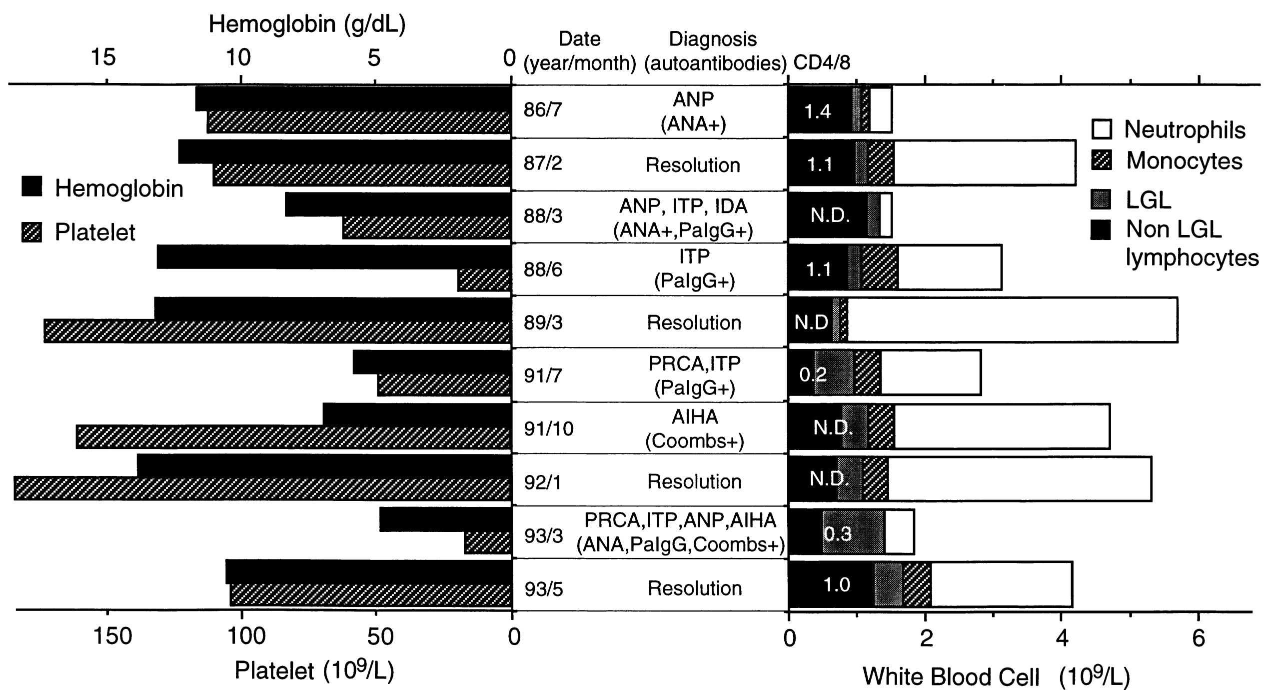

Fig 1. Changes of blood cell counts during clinical course of the patient. ANP: autoimmune neutropenia; ITP: idiopathic thrombocytopenic purpura; PRCA: pure red cell aplasia; AIHA: autoimmune haemolytic anaemia; ANA: anti-neutrophil antibody; PaIgG: platelet-associated IgG; N.D.: not determined. CD4/8 is the ratio of blood $CD4^+$ to $CD8^+$ cells within a lymphocyte gate on flow cytometric analyses. This gate includes both LGL and non-LGL lymphocytes.

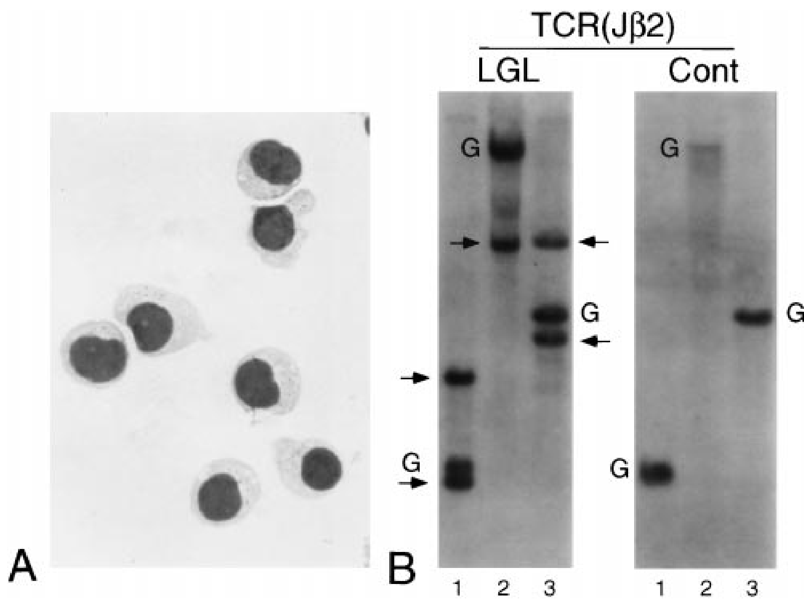

Fig 2. (A) Cytocentrifuged smear of $CD8^{+}$ cells enriched by immunomagnetic beads in April 1993. Cells bear the morphology of mature large granular lymphocytes (Giemsa staining, ×1000). (B) Southern blot analysis of TCR(Jβ2) gene in $CD8^{+}$ LGL cells. The TCR gene was clonally rearranged. Restriction enzymes used were EcoRI (1), BamHI (2), HindIII (3). Human placenta was used as a negative control (Cont). G: germline bands; arrows: rearranged bands.

of total bone marrow cells) and blood reticulocytes were undetectable (<1‰). A high level of platelet-associated IgG was also seen. The absolute number of blood lymphocytes was normal. However, we found that 65% of lymphocytes were $CD8^{+}$ cells, and 61% (640/μl) of blood lymphocytes were morphologically LGL (Fig 2A). Southern blot analysis of *TCRB* (Jβ2) genes of purified $CD8^{+}$ cells revealed that these cells were of monoclonal origin (Fig 2B). Based on these data, we diagnosed this patient to have T-LDGL. The administration of cyclosporine A (2 mg/kg) resulted in the partial resolution of both ITP and PRCA that was associated with a decrease in LGL numbers. On October 1991 the patient presented AIHA with reticulocytosis (61‰). The bone marrow was hypercellular with 32% erythroid cells, the serum haptoglobin was low (<0·01 mg/l), and the indirect bilirubin was elevated (35·7 μmol/l). The direct Coombs test was positive, and the presence of serum warm-active IgG antibodies against erythrocytes was indicated by an indirect Coombs test. The absolute number of LGL cells was $0{\cdot}66\times10^{9}$/l. Cyclosporine A (3 mg/kg) was effective in treating the AIHA. The number of blood $CD3^{+}8^{+}$ large granular lymphocytes decreased to $0{\cdot}34\times10^{9}$/l.

In April 1993 the patient presented with pancytopenia. There was an increase of $CD3^{+}8^{+}$ LGL ($0{\cdot}9\times10^{9}$/l). The patient suffered from PRCA, AIHA, ITP and autoimmune neutropenia. The detailed surface phenotypes of blood mononuclear cells within lymphocyte scatter gates at this time were: CD2: 91%, CD3: 90%, CD4: 12%, CD8: 75%, CD5: 89%, CD7: 86%, CD13: <0·5%, CD14: <0·5%, CD16: 2·7%, CD19: <0·5%, CD20: <0·5%, CD25: 1·6%; CD33: <0·5%, CD34: <0·5%, CD56: 0·5%, CD57: 65%, TCR$\alpha\beta$: 90%, TCR$\gamma\delta$: 4%, HLA-DR: 63%. Two-colour flow cytometric analysis showed that the majority of blood lymphocytes were $CD3^{+}8^{+}$ (75%) and $CD3^{+}$TCR-$\alpha\beta^{+}$ (85%). Epstein-Barr virus DNA was not detected. Circulating LGL had a chromosomal abnormality of $20q^{-}$, indicating that LGL might be neoplastic, but not reactive cells. Cytoreductive chemotherapy for the clonal LGL cells was not applicable because of severe pancytopenia. Cyclosporine A was transiently effective, but the patient gradually became refractory to cyclosporine A as well as steroids. The number of LGL cells remained $<1\times10^{9}$/l. Pancytopenia persisted, and she died of a brain haemorrhage in July 1997.

During the course of the disease other autoantibodies such as anti-rheumatoid factor, antinuclear antibodies and anti-DNA antibodies were not detected. Since $CD8^{+}$ LGL have been shown to occasionally suppress *in vitro* growth of erythroid progenitors (Oshimi *et al*, 1988), we evaluated the effect of the patient's LGL on the growth of bone marrow progenitors by a method previously reported (Taniguchi *et al*, 1988). The purified $CD2^{+}$ T cells (in July 1986 and June 1991) or $CD3^{+}8^{+}$ LGL (April 1993) did not inhibit either erythroid, megakaryocyte or granulocyte/macrophage colony formation from bone marrow mononuclear cells in the patient and normal controls (data not shown).

DISCUSSION

This patient presented a variety of autoimmune cytopenias over an 11-year clinical course. The purified clonal LGL did not directly inhibit the growth of myeloerythroid progenitors, at least *in vitro*. Because independent autoantibodies to neutrophils, platelets and erythrocytes appeared throughout the 11 years of the patient's clinical course, and because all of the autoimmune haemopoietic disorders were transiently controlled by T-cell-targeted immunosuppressive therapy with cyclosporine A, the deregulated B-cell immunity may have been the result of the presence of clonal neoplastic $CD3^{+}8^{+}$ TCR$\alpha\beta^{+}$ LGL.

It remains unknown how clonal LGL frequently cause autoantibody production from B cells against haemopoietic cells. Inhibition of normal T-cell development and its functions by dominant proliferation of LGL may be critical for the autoantibody production from B cells. In this context,

the presence of circulating clonal LGL $>2\times10^9$/l, the standard criteria for the diagnosis for LDGL (Loughran, 1993), may usually be required to cause the deregulation of B-cell immunity that leads to autoimmune haemopoietic disorders. In this patient the absolute number of LGL cells during the first 5 years of episodic autoimmune cytopenias was normal, and a relative increase in the percentage of blood $CD8^+$ LGL led us to test the clonality of this population. Our results suggest that the presence of a small population of neoplastic $CD3^+8^+$ $TCR\alpha\beta^+$ LGL could cause various autoimmune haemopoietic disorders. Analysis of CD4/CD8 ratio and/or $V\beta$ repertoire of T cells (Semenzato *et al*, 1997) might help in detecting clonal LGL of T-cell type. Physicians caring for patients with autoimmune cytopenia should be aware of insidious T-LDGL.

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