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Immunological Functions and T-Cell Receptor Gene Rearrangement of Proliferating Lymphocytes in a Case of T Gamma Lymphocytosis with Neutropenia

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OKABE, M., TANAKA, M., UEHARA, Y., MATSUSHIMA, S., KAKINUMA, M., ABE, F., KOBAYASHI, M., TACHIBANA, N., SAKURADA, K., SAITO, H. and MIYAZAKI, T. *Immunological Functions and T-Cell Receptor Gene Rearrangement of Proliferating Lymphocytes in a Case of T Gamma Lymphocytosis with Neutropenia.* Tohoku J. *exp.* Med., 1987, **151** (1), 105-115 —— A case of Tγ lymphocytosis with neutropenia is presented. The patient showed mild lymphocytosis, splenomegaly, anemia, neutropenia and recurrent infections without progression for 15 years. The expanded lymphoid cells were morphologically large granular lymphocytes (LGL), had receptors for both sheep red blood cells and IgG-Fc portion and were positive for OKT3 and 8 antigens. They displayed ADCC activity, whereas they showed low responses to T-cell mitogens and deficient NK activity. They showed neither suppressor activity on antibody production by B-cells nor suppressor activity on CFU-C formation. The DNA isolated from the expanded cells of the patient showed T-cell β-chain (Tβ) gene rearrangement, indicating monoclonality of the proliferation. This finding supports that the proliferation of T8 lymphocytes in the present case is neoplastic rather than reactive, regardless of the benign clinical course. ———— Tγ lymphocytosis with neutropenia; large granular lymphocytes (LGL); ADCC activity; T-cell receptor gene

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Tγ lymphocytosis with neutropenia is a rare chronic lymphoproliferative disorder characterized by moderate splenomegaly, lymphocytosis with bone marrow infiltration and neutropenia (McKenna et al. 1977; Bom-van Noorloos et al. 1980; Linch et al. 1981). The proliferating cells in this disorder are morphologically mature lymphocytes with an abundant cytoplasm containing numerous azurophilic granules, and they have been identified phenotypically as Tγ lymphocytes or T8 lymphocytes. Therefore, this disease is distinct from any other previously described T-cell malignancies such as adult T-cell leukemia (ATL), T-cell ALL and peripheral T-cell lymphomas (Aisenberg et al. 1981; Linch et al. 1981). At present, most authors prefer to classify this disorder as a reactive one rather than a neoplastic one, since the disease follows a chronic course (Reynolds and Foon 1984; Minden et al. 1985; Toki et al. 1985).

The difficulty of discriminating the neoplastic from the reactive nature of this disorder has been brought about by the lack of an adequate method to probe clonal proliferation of T cells. Recently, the isolation and the characterization of DNA clones corresponding to the T cell beta-chain receptor (Tβ) gene has demonstrated that the Tβ genes undergo rearrangements during T cell differentiation analogous to immunoglobulin gene (Ig) rearrangements in B cells (Hedrick et al. 1984; Yanagi et al. 1984). Therefore, the analysis of T cell receptor gene rearrangement offers a powerful means to analyze monoclonality of T-cell proliferation.

In the present study, we investigated the immunological functions and the Tβ gene configuration in proliferating lymphocytes obtained from the peripheral blood of a patient with Tγ lymphocytosis with neutropenia in order to clarify the pathophysiology of the disorder.

Case Report

A 37-year-old man with a 15-year history of anemia was transferred to our hospital for further evaluation of anemia. For 2 years since then, he has had recurrent infections, icterus and gradual increase of hepatosplenomegaly. On admission, intensive anemia and icterus were noted. No enlargement of the lymph node was detected, but moderate hepatosplenomegaly was noted; the liver edge was palpable 7 cm below the right costal margin and the spleen edge 9 cm below the left costal margin.

Laboratory findings showed normocytic and normochromic anemia with reticulocytosis (red blood cell count; 185×10^{10}/liter, Hb 6.1 g/100 ml, Ht 17.7%, reticulocytes 3.4%), platelet count of 13.5×10^{10}/liter and white blood cell count of 7.3×10^{9}/liter. The differential count of white blood cells showed moderate but absolute lymphocytosis of 6.5×10^{9}/liter with severe granulocytopenia of 0.56×10^{9}/liter. The increased lymphocytes were mature and uniformly lage, 12–15 μm in diameter, and had a well-developed cytoplasm with numerous azurophilic granules, which were compatible with the appearance of large granular

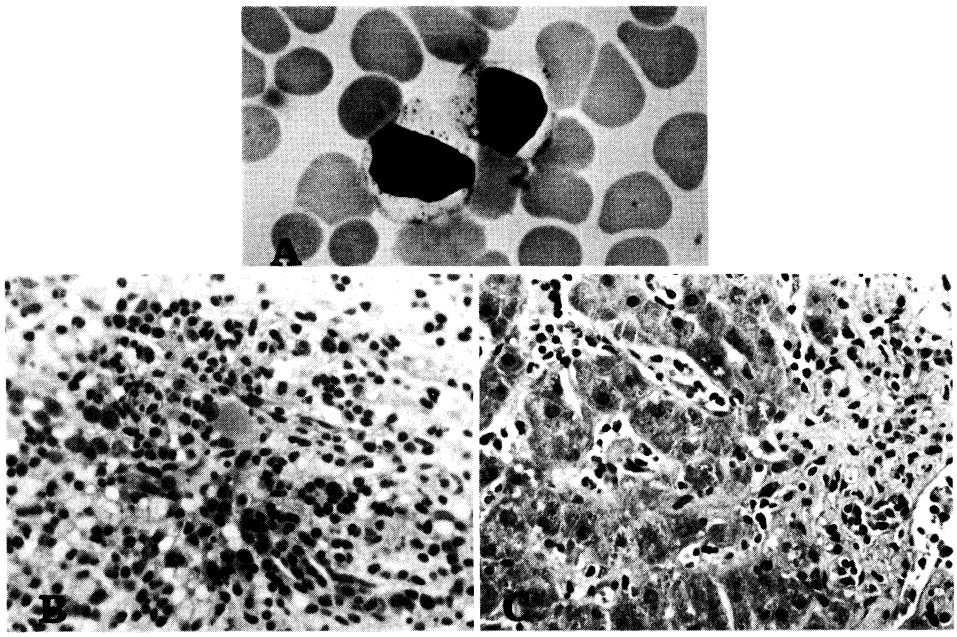

Fig. 1. A : Large granular lymphocytes in peripheral blood stained with May-Grünwald-Giemsa. B : Infiltration of mature lymphocytes in bone marrow (H-E stain). C : Sinusoidal and portal infiltration of mature lymphocytes in liver (H-E stain).

lymphocytes (LGL) (Timonen et al. 1981) (Fig. 1A). The biochemical findings showed elevated serum lactic dehydrogenase (isoenzymes I and II were dominant), slight hyperbilirubinemia due to elevation of indirect bilirubin and marked diminution of serum haptoglobin. Direct Coombs test was weakly positive. Ferrokinetic study showed shortness of plasma iron disappearance (PID t1/2) and gradual increase of radioactivity in the spleen. These findings suggested the presence of hemolytic anemia which might be caused by an autoantibody. No other autoantibodies were deteceted. Moderate hypogammaglobulinemia was noted ; IgG 611 mg/100 ml, IgA 44 mg/100 ml, and IgM 137 mg/100 ml.

Bone marrow biopsy revealed normocellular marrow and infiltration with atypical mature lymphocytes (Fig. 1B). Liver biopsy also revealed sinusoidal and portal infiltration with similar mature lymphocytes (Fig. 3C).

Chromosomal analysis with a Q banding technique of a 3 day cultivation of bone marrow mononuclear cells and peripheral blood cells showed the normal karyotype, 46, XY. ATL, which is endemic in southwest Japan, was ruled out on the basis of its unique clinical features and the negativism of serum antibodies to ATL-associated antigens (ATLA) (Uchiyama et al. 1977 ; Hinuma et al. 1981). A diagnosis of chronic Tγ lymphocytosis with neutropenia was made and the patient was given prednisolone. At present, lessening of anemia and gradual decrease of hepatosplenomegaly and peripheral blood lymphocytosis indicate

TABLE 1. *The phenotypical analysis of the*

	E-RFC	smIg	Fcr	OKT3
Peripheral blood	73.0	1.0	44.0	98.1
Bone marrow	NT	1.0	NT	43.2

NT : not tested.

improvement of the disease, but neutrophil counts in peripheral blood have not shown recovery.

MATERIALS AND METHODS

Cell marker analysis

Cell surface markers were analyzed by an Epics C flow cytometery (Coulter Electronics, Hialeah, Fla., USA) using commercially available monoclonal antibodies and FITC-labeled $F(ab')_2$ fragment of goat anti-mouse Ig antibody (Cappel Lab., Cochranville, PA, USA). The monoclonal antibodies, OKT3, 4, 8, 11, OKIa1 and OKM1, were purchased from Ortho

TABLE 2. *The immunological functions of the proliferative lymphocytes in the present case*

1) Cytotoxic activities

E : T ratio	NK activities (%)		ADCC activities (%)	
	Patient	Control	Patient	Control
50 : 1	2.4	NT	NT	NT
25 : 1	0.0	28.9	69.0	41-72

2) Mitogen responses

Mitogens	Stimulation index (mean±S.E.)	
	Patient	Control
PHA-P (2 μg/ml)	25.5±0.4	161.2±9.6
Con A (10 μg/ml)	16.3±1.2	69.1±3.3

3) Inhibition of immunoglobulin synthesis by normal lymphocytes when co-cultured with proliferative lymphocytes of the patient

Producer cells	Added E-RFC	Ig synthesis (ng/ml)/(% inhibition)		
		IgM	IgG	IgA
PBL of healthy donor	Non	3400	1400	3400
	Healthy donor E-RFC	4300 (−26%)	1500 (− 7%)	4900 (−44%)
	Patient's E-RFC	4000 (−17%)	1900 (−36%)	4300 (−26%)

NT : not tested.

proliferative lymphocytes in the present case

OKT4	OKT8	OKT11	OKIa1	Leu7	IL-2R
14.7	69.7	89.5	79.6	1.3	3.0
6.1	33.9	41.4	NT	NT	NT

Pharmaceutical Cooporation (Raritan, NJ, USA), and the monoclonal antibodies, Leu7 and Leu IL-2R were purchased from Becton Dickinson (Sunnyvale, CA, USA).

E-rosette-forming cells (E-RFC) were asseyed by the method of van Oers et al. (1977)

Cytotoxicity assays

Natural killer (NK) activity was assayed by ^{51}Cr-release using K562 cell line as target cells, as previously described in detail (Okabe et al. 1986). Antibody dependent cell-mediated cytotoxicity (ADCC) activity employing ^{51}Cr-labeled chicken red blood cells as target cells was assayed by the method of Saksela et al. (1977).

Responsiveness to T-cell mitogens

The proliferative responses of lymphocytes to T cell dependent mitogens were assayed by adding 2 μg/mL PHA-P or 10 μg/ml concanavaline A (ConA ; Wellcome Research Lab., Beckenham, England, UK) and adding 0.5 μCi of ^{3}H-thymidine per well on the third day of cultivation, as previously described (Okabe et al. 1986).

Supperessor activity on immunoglobulin synthesis

Suppressor activity on immunoglobulin synthesis was assayed by the method of Waldmann et al. (1974). Separation of E-rosette forming cells (E-RFC) was carried out by the method of van Oers et al. (1977). Briefly, aliquots of PBL (2×10^{6}/ml) were mixed with an equal volume of neuraminidase-treated SRBC (2×10^{8}/ml), centrifuged for 5 min at $200\times$G, and incubated at 4°C for 90 min. The rosettes were resuspended and layed on a Ficoll-Conray cushion and centrifuged at room temperature. E-RFC were recovered from the pellets. PBL (5×10^{5}/ml) from normal individuals were incubated with E-RFC (10^{5}/ml) obtained from the patient or normal controls in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 μg/ml kanamycin and 100 U/ml penicillin G. After a 7-day culture with 10 μg/ml of pokeweed mitogen (Gibco, Grand Island, NY, USA) at 37°C in a humidified atmosphere of 5% CO_2, a supernate of the culture was harvested for measuring the level of IgG, IgA and IgM by an enzyme-linked immunosorbent assay (ELISA),

TABLE 3. *The effect of proliferative lymphocytes to CFU-C formation*

	Number of CFU-C colony	
	The patient	Healthy donors ($n=16$)
Bone marrow cells	31± 3	101.6 ±58.3
Adherent cells-depleted bone marrow cells	42± 8	NT
E-rosette forming cells-depleted bone marrow cells	62±15	NT
E-depleted bone marrow cells +peripheral E-rosette forming cells	67±24	NT

NT : not tested.

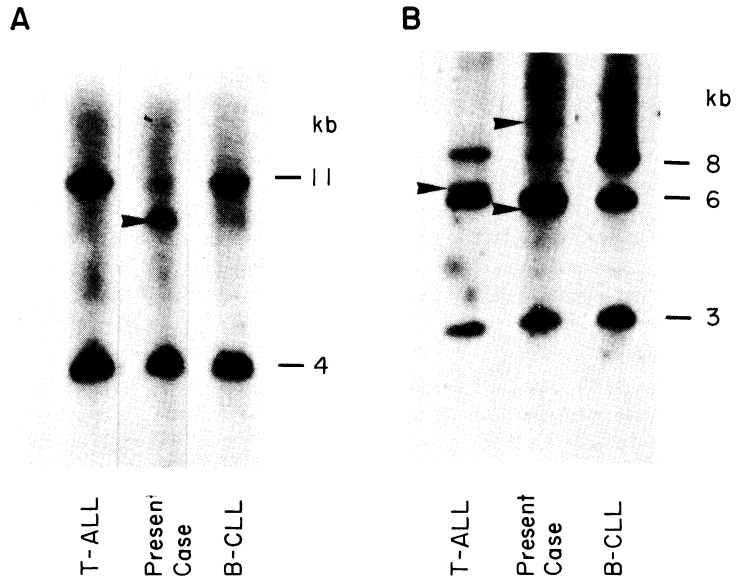

Fig. 2. Tβ gene configurations in DNA isolated from the expanded lymphocytes of peripheral blood. A : The rearrangement patterns of Tβ genes in *Eco* RI-digested DNA. B : The rearrangement patterns of Tβ genes in *Hind* III-digested DNA. Rearranged genes are indicated by marks (▶).

previously described in detail by Ariga et al. (1985). Suppressor activity was calculated by the following formula : % Inhibition = (1 − Synthesis of Ig by cells in coculture (ng/ml)/ Synthesis of Ig by PBL of healthy donor) × 100(%)

CFU-C assay

CFU-C assay was performed by plating 2×10^5 bone marrow cells using the agar method of Pike and Robinson (1970). Cell aggregates containing greater than 40 cells were judged as a colony.

T-cell receptor β-chain analyses

High molecular weight DNA was extracted from the cells according to the method of Perucho et al. (1981) with minor modification (Koda et al. 1985). Ten to 20μg of DNA were digested to completion with either *Eco* RI or *Hind* III, electrophoresed through 0.75% agarose gels and transferred to nitrocellulose membranes. The membranes were hybridized with nick-translated DNA probe of Tβ, washed 5 times with a solution containing 0.3 × SSC (45 mM sodium chloride plus 4.5 mM sodium citrate) and 0.1% (W/V) SDS at 65°C, and autoradiographed at −80°C with an intensifying screen for several days. A plasmid containing human Tβ cDNA (JUR-β2) (Yoshikai et al. 1984) which span from 9 bases 5′ of J segment to poly A tract was kindly provided by Dr. T.W. Mak, and its 0.85 kb *Pst* I fragment was used as the Tβ probe.

Results

Surface markers of proliferative lymphocytes

Surface markers of the patient's lymphocytes are presented in Table 1. They

showed E-RFC$^+$, OKT11$^+$, OKT3$^+$, 4$^-$, 8$^+$, and IgG-Fc receptor$^+$, and they lacked OKM1 and Leu7 antigens, which are present on normal LGL. Although they were positive for OKIa1, they were negative for Leu IL-2R.

Analyses of immunological functions

Immunological functions of increased lymphocytes are presented in Table 2. They showed diminished NK activity and hyporesponsiveness to T-cell mitogens, PHA-P and Con A, but retained a normal level of ADCC activity. They showed no suppressor activity on the polyclonal immunoglobulin production by B lymphocytes stimulated with pokeweed mitogen.

The effects of expanded lymphocytes on granuloid colony formations

The number of CFU-C colonies of the unfractionated bone marrow cells obtained from the patient was slightly diminished as compared with that of healthy donors. Removal of E-rosette forming cells did not increase CFU-C colony formation significantly. The addition of an equal number of peripheral E-rosette forming cells obtained from the patient did not suppress CFU-C formation of E-RFC- depleted bone marrow cells (Table 3).

T cell receptor gene configurations

A 0.85 kb Pst I-fragment of JUR- β2 cDNA, which hybridizes with both Cβ1 and Cβ2 (Yoshikai et al. 1984), was used. *Eco* RI-digested germ-line T region genes gave 2 hybridization signals, 11 kb for Cβ1 and 4 kb for Cβ2 (Fig. 2A), and *Hind* III -digested germ-line T region genes gave 3 hybridization bands of 8 and 6 kb for Cβ2 and 3 kb for Cβ1 (Fig. 2B) in the leukemic cells isolated from a B-CLL patient. The patterns of germ-line arrangement of T genes were consistent with those described in Waldmann's study (1985). A T-ALL case showed the rearranged Cβ2 gene in *Hind* III-digested DNA (Fig. 2B). The present case showed a faint 11 kb band of Cβ1 which may be derived from the normal PBL other than the proliferating LGL, and showed the appearance of a new 9 kb band in *Eco* RI-DNA (Fig. 2A). In the *Hind* III-digested DNA, 6 kb band of 3′ Cβ2 showed a doublet formation and 8 kb band of 5′ Cβ2 showed decreased intensity with the appearance of a new band. These results indicate that the proliferating LGL of the present case contains the rearranged Cβ1 on one alle with deletion of the other alle, and the rearranged Cβ2 on one alle with the germ-line arrangement of the other alle, which support the clonal nature of the proliferating LGL.

Discussion

We describe here a rare case of chronic T-cell lymphocytosis (T gamma lymphocytosis with neutropenia) who manifested peripheral blood lymphocytosis characteristic of LGL, bone marrow infiltration and mild splenomegaly without

involvement of lymph nodes. Similar to the clinical observations of our case, LGL proliferation of T gamma lymphoproliferative disease are generally characterized by a relatively low WBC count, an indolent, nonprogressive clinical course and prolonged survival. The benign clinical course in most patients has led some authors to question the neoplastic origin of the disease and to recommend the term chronic T-cell lymphocytosis unless progression of the disease is demonstrable (Newland et al. 1984 ; Semenzato et al. 1984 ; McKenna et al. 1985).

The expanded lymphocytes of our case showed the morphological characteristics of LGL, and they shared surface markers with suppressor/cytotoxic T lymphocytes such as OKT3$^+$, 8$^+$ and IgG-Fc-receptor$^+$ but did not share surface markers with NK cells such as Leu7 and OKM1. Functionally, they exhibited ADCC activity but no NK activity. These findings suggest the view that the expanded LGL in the present case originated not from NK cells but from a cytotoxic/suppressor subpopulation of T lymphocytes, which conflicts sharply with the recent view of Pandolfi (1985) that the expanded granular lymphocytes in chronic T-cell lymphocytosis originate from NK cells.

In spite of the existence of T-cell markers, the lymphocytes of the present case showed deficient responses to T-cell mitogens PHA and ConA, and showed no suppressor activities on the polyclonal immunoglobulin production by B cells stimulated with pokeweed mitogen. The mechanism of T-cell function deficiency in the expanded lymphocytes is not clear at present. One possible explanation is that the lymphocytes may have originated not from a subset of suppressor T-cells but from a subset of cytotoxic T-cells, and another possibility is that it may be related to the neoplastic process.

Several mechanisms of neutropenia in Tγ lymphocytosis have been discussed. Several authors described that autoantibodies against granulocytes may involve neutropenia via ADCC of Tγ cells or a directory effect (Bom-van Noorloos et al. 1980 ; Starkebaum et al. 1983), while no antibody was detected in the present study (data not shown). We investigated the effect of the proliferating lymphocytes on granuloid colony formation (CFU-C), but could not demonstrate their inhibitory effect on CFU-C formation. However, this finding may not simply exclude the inhibitory effect of the proliferating Tγ cells on myelopeiesis, since CFU-C was assayed in a condition of impossible contact of the Tγ cells with the myeloid progenitor cells in agar.

The determination of monoclonality within lymphoid neoplasms has been restricted mainly to B-cell tumors that display the exclusive presence of one immunoglobulin light-chain isotype, κ or λ. The lack of an adequate marker of clonality for T-cell and poor proliferative response to T-cell mitogens, which is essential for chromosome analysis, has brought controversy regarding the neoplastic or reactive nature of this disorder. However, the detection of the Tβ gene rearrangement has been recognized as a powerful approach to identify clonal proliferation of the T-cell lineage cells as well as to identify the different stages of

development of T cells in ALL (Flug et al. 1985; Minden et al. 1985). More recently, several authors have reported analyses of Tβ gene rearrangements in chronic T lymphocytosis (Aisenberg et al. 1985; Bertness et al. 1985; Waldmann et al. 1985; Foa et al. 1986) and demonstated clonal rearrangements in most cases. We also have investigated the Tβ gene rearrangement in DNA isolated from the proliferative lymphocytes of the present case and have detected the clonal rearrangement of TCβ gene. This clonal rearrangement of Tβ gene argues against the disease being a reactive disorder and supports the clonal nature of T-cell proliferation. The present study suggests that Tγ lymphocytosis with neutropenia might belong to a benign variant of T-CLL. But, the present case has not gotten into a progressive state for a long time, in spite of the presence of the monoclonal rearrangement of Tβ gene. Thus, the monoclonality of it does not simply imply malignancy. Therefore, a long term study including detailed clinical observations and analyses of Tβ genes and oncogenes may be necessary to resolve the pathophysiology of Tγ lymphocytosis with neutropenia.

Acknowledgments

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