T CELL RECEPTOR β CHAIN GENE REARRANGEMENTS IN LYMPHOPROLIFERATIVE DISORDERS OF LARGE GRANULAR LYMPHOCYTES/NATURAL KILLER CELLS

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Approximately 10% of circulating peripheral blood lymphoid cells belong to the large granular lymphocyte (LGL) population. LGL exhibit characteristic cytomorphology, histochemical properties, and monoclonal antibody–defined cell surface markers (reviewed in 1 and 2). They mediate natural killer (NK) activity and antibody-dependent, cell-mediated cytotoxicity (ADCC) and may act as a first line of resistance against foreign cells, microorganisms, and tumor cells (1, 2). The lineage derivation of LGL is unclear and has not been conclusively identified by phenotypic and functional studies. Several reports (1, 2) have shown that LGL are heterogeneous and share characteristics of both T lymphocytes (e.g., T11, T10, T8 expression and interleukin 2 [IL-2] responsiveness) and mononuclear phagocytes (e.g., Mol expression, IL-1 production, and cytotoxic activity of broad specificity against tumor cell lines). It has also been suggested that LGL may belong to a separate lineage (3).

Rarely, pathologic proliferations occur in humans in cells displaying several features of LGL, including morphology, cytochemistry, NK-related cell surface markers, high ADCC, and, in some cases, spontaneous NK activity (for review see 4). These LGL expansions have been variously referred to as chronic Tγ cell leukemia, Tγ lymphocytosis, or Tγ lymphoproliferative disease (Tγ LPD [4]). We investigated (5) the organization of the gene coding for the beta chain of the T cell receptor (Tβ) in LGL expansions occurring in 12 patients with Tγ LPD. Based on the fact that the Tβ gene rearranges early during the T cell differentiation pathway (6), our study was aimed at elucidating the relationship between LGL/NK and T cells in terms of lineage derivation and immune function.

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Materials and Methods

Patients and Cell Samples. All 12 patients exhibited peripheral blood and bone marrow infiltration by cells displaying characteristic LGL morphology, i.e., abundant moderately basophilic cytoplasm, numerous azurophilic granules, round nuclei, and absent nucleoli. LGL were isolated from 12 patients with Tγ LPD by Ficoll-Hypaque density gradient centrifugation of freshly drawn heparinized peripheral blood, followed by depletion of adherent monocytes on plastic. Normal LGL were obtained from healthy donors as described by Timonen and Saksela (7) on a discontinuous 35–47.5% gradient of Percoll (Pharmacia, Inc., Uppsala, Sweden), with minor modification.

Monoclonal Antibody–defined Cell Surface Markers and Morphology. The membrane phenotype of the isolated cells was determined by indirect immunofluorescence using a Zeiss microscope and/or a FACS IV (Becton Dickinson Immunocytometry Systems, Mountain View, CA). The following panel of monoclonal antibodies was used: OKT3, OKT6, OKT8, OKT11, and OKM1 (Ortho Diagnostic Systems Inc., Raritan, NJ or gifts of Dr. E. Reinherz, Dana-Farber Cancer Institute, Boston, MA); HNK-1 (8), reactive with NK cells and a subset of T cells (courtesy of Dr. T. Abo, University of Alabama, Birmingham, AL); B73.1 (2) (courtesy of Dr. G. Trinchieri, Philadelphia, PA) and AB8.28 (9) (courtesy of Dr. Malavasi, University of Torino, Torino, Italy), both reactive with a structure closely associated with the Fcγ receptor of LGL; N901, reactive with LGL and some myeloid precursors (10) (courtesy of Dr. J. Griffin, Dana-Farber Cancer Institute, Boston, MA).

Cytotoxicity Assays. NK activity was assessed by using the K562 cell line at effector/target cell (E/T) ratios of 25:1 to 100:1, in a 4 h ^{51}Cr-release assay, as previously described (11). For ADCC, ^{51}Cr-labeled TLX9 murine lymphoma cells, sensitized with 1:10,000 rabbit antibody, were used as targets in a 4 h test (12).

In a series of experiments, effector cells (2×10^6/ml in RPMI 1640 medium with 10% pooled human serum) were cultured for 72 h with IL-2. Supernatant of the gibbon lymphosarcoma line MLA144 (50% final concentration, 100 U/ml) (13) or recombinant IL-2 (100 μg/ml) (a gift of Dr. J. Schindler, Biogen Research Corp., Cambridge, MA) were the IL-2 sources. Cytotoxicity was assessed against K562 cells or against the relatively NK-resistant Daudi line. We will refer to IL-2-induced cytotoxicity as lymphokine-activated killer (LAK) function, as suggested by others (14). Results are presented as the percentage of specific lysis (mean ± SD, three replicates per group) after subtraction of spontaneous release in the absence of effectors. 4–6% differences above background were statistically significant.

DNA Extraction and Southern Blot Analysis. DNA was prepared by cell lysis, proteinase K digestion, extraction with phenol, and precipitation with ethanol (15). 15 μg of DNA were digested with the appropriate restriction endonuclease, electrophoresed in a 0.8% agarose gel, denatured, neutralized, transferred to a nitrocellulose filter, and hybridized according to Southern (15). Filters were washed in 0.2 × SSC, 0.5 sodium dodecyl sulfate (SDS), pH 7 at 60°C for 2 h.

DNA Probes. The Tβ probe was derived from a human cDNA Tβ clone (YTJ-2) isolated from the Jurkat-2 T lymphoma cell line (courtesy of Dr. Tak Mak) (5). This clone hybridizes to both alleles ($C_{\beta 1}$ and $C_{\beta 2}$) of the constant region and to one or more alleles of the variable regions (5, 16). The generation of fragments specific for the constant (TβC) region have been previously described (16). DNA fragments were ^{32}P-labeled by nick translation for use as probes (15).

Results

Table I summarizes the principal immunophenotypic and functional properties of the LGL isolated from each of the 12 cases of Tγ LPD. In each case, the LGL expressed the T11 sheep erythrocyte receptor, the NK cell surface marker HNK-1, and Fcγ receptors identified by rosetting techniques (data not shown), by AB8.28 or by B73.1 monoclonal antibodies, and by ADCC effector function.

TABLE I

Phenotypic, Functional, and Immunogenotypic Analysis of LGL from T_γ LPD Cases

No.	Phenotypic markers									Cytotoxicity*				Tβ gene‡
	T11	T3	T6	T8	HNK1	OKM1	B73.1	AB8.28	N901	NK K562	ADCC TLX9	LAK K562	LAK Daudi	
1	85	21	0	71	48	NT§	NT	NT	NT	NT	NT	NT	NT	G
2	82	4	0	2	65	75	17	NT	NT	54.5 ± 1.3	24.3 ± 2.1	NT	NT	G
3	75	91	0	10	75	1	1	40	1	3.6 ± 0.3	54.4 ± 1.6	55.1 ± 2.5	65.8 ± 7.4	R
4	89	78	0	54	93	45	50	4	33	69.0 ± 3.3	53.6 ± 1.7	74.8 ± 4.0	69.1 ± 7.1	R
5	91	98	NT	92	89	3	12	25	10	3.7 ± 1.6	25.2 ± 5.6	25.1 ± 1.9	9.4 ± 2.5	R
6	88	92	0	88	75	5	60	57	0	7.4 ± 1.0	34.1 ± 4.2	80.1 ± 8.0	27.5 ± 6.9	R
7	80	85	0	50	79	62	3	73	85	13.3 ± 5.6	40.4 ± 9.4	51.7 ± 5.3	61.8 ± 6.8	R
8	93	90	0	67	63	53	11	9	4	4.7 ± 1.6	28.0 ± 5.6	47.2 ± 3.6	45.8 ± 6.7	R
9	93	90	1	39	42	30	29	15	12	14.0 ± 5.1	58.5 ± 10.6	4.6 ± 1.1	0.4 ± 1.6	R
10	75	80	0	32	71	61	5	57	53	2.8 ± 0.1	52.4 ± 11.5	23.9 ± 4.9	50.3 ± 3.8	R
11	66	81	0	44	55	28	25	20	31	26.3 ± 3.5	25.1 ± 2.5	39.5 ± 2.4	25.5 ± 3.9	R
12	90	81	NT	52	90	20	12	52	8	17.8 ± 2.2	31.6 ± 5.0	67.9 ± 2.6	18.1 ± 2.1	R
Normal LGL range¶	65–75	1–4	0–2	25–35	60–70	60–70	65–75	60–70	55–65	18.8–52.6	13.6–54.0	24.1–58.6	51.0–66.9	

Cells were cultured with IL-2 for 72 h, except those from patient No. 12, which were tested after 24 h. Cells cultured in medium alone had no appreciable cytotoxic activity, except for patient 12, whose cells showed 47.9 ± 4.0% specific lysis against K562 and no activity against Daudi.

* Results are expressed as mean ± SD and refer to an E/T ratio of 50:1.

‡ G, Germline configuration; R, rearranged.

§ NT, not tested.

¶ Results are expressed as the range of 20 preparations of Percoll-enriched LGL (60–85% pure by morphology).

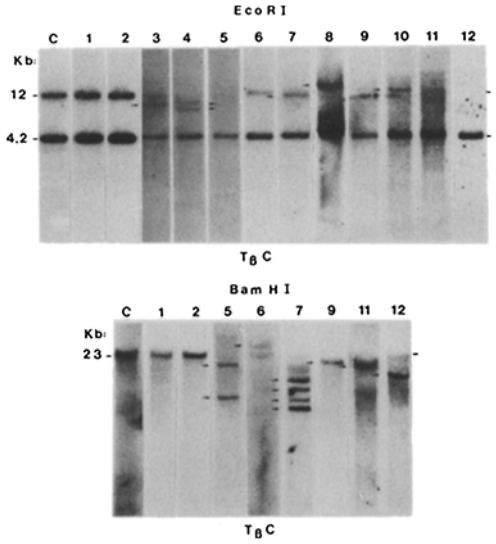

FIGURE 1. Tβ gene rearrangements in Tγ LPD cases. DNA was extracted from LGL derived from the same Tγ LPD cases illustrated in Table I. DNA were digested with the indicated restriction enzymes and hybridized to a Tβ probe representative of the constant region of the Tβ gene (TβC).

Surface markers T3, T8, OKM1, and N901 were detected in the LGL isolated from some but not all cases. All LGL were negative for T6. LGL from all Tγ LPD patients had high ADCC, whereas high NK activity was only detected in cases 2, 4, and 11. LGL from Tγ LPD (except patient 9), on cultivation in the presence of IL-2 for 72 h, developed high lytic activity against both K562 and the more resistant Daudi cells. LGL cultured in medium alone showed some enhancement of lytic activity (up to 40% specific lysis against K562) in one case (No. 12).

We analyzed the organization of TβC by Southern blot hybridization using Eco RI or Bam HI restriction enzymes, and a TβC probe. Results are summarized in Table I and illustrated in Fig. 1. The cells isolated from nine cases exhibited bi-allelic deletion of the Eco RI 12.0 kb DNA fragment, a pattern we have previously shown to be characteristic of both polyclonal and monoclonal immature and mature T cell populations (16). Clonal rearrangements of the TβC gene were demonstrated in five of these nine cases, indicated by the presence of new hybridization bands that were not detectable in control DNA (see bands marked by arrow in cases 3, 4, 7, and 10 in Fig. 1). In the remaining four cases (Nos. 5, 9, 11, and 12), clonal rearrangements were found when the extracted DNA were digested with Bam HI, thus demonstrating the monoclonal nature of all nine cases of Tγ LPD. The LGL isolated from the three remaining cases (Nos. 1, 2, and 6) displayed the germline Eco RI band pattern. However, Bam HI digestion demonstrated the presence of clonally rearranged bands in case 6. The remaining two cases (Nos. 1 and 2) displayed an intact germline TβC locus upon digestion with Eco RI, Bam HI (see Fig. 1), and Hind III (not shown). It is noteworthy that these two Tβ gene–unrearranged cases did not appreciably express the T3 antigen (Table I). We conclude that Tγ LPD are heterogeneous in the organi-

zation of the Tβ gene. The more frequently encountered cases of $T3^+$ Tγ LPD show clonal rearrangements ($T3^+T\beta^+$ Tγ LPD), whereas the less common $T3^-$ Tγ LPD exhibit the germline Tβ configuration ($T3^-T\beta^-$ Tγ LPD).

Discussion

Our results appear to divide the 12 cases of Tγ LPD into two distinct groups, based on their expression of the T3 antigen and on rearrangements of the Tβ gene. Both $T3^+T\beta^+$ and $T3^-T\beta^-$ Tγ LPD usually expressed and, in most instances, coexpressed, the T cell–restricted antigens T11 and T8 and the myeloid-associated antigent Mol. Most notably, an analogous pattern was found in clonal NK populations derived from normal cells; i.e., both $T3^+T\beta^+T\alpha^+$ and $T3^-T\beta^-T\alpha^-$ NK clones exist and both groups are heterogeneous in the expression of the T8 and Mol antigens (18, 19). These observations indicate that the immunogenotypic and immunophenotypic heterogeneity of these Tγ LPD cases is not due to lineage infidelity, a phenomenon occasionally observed in hematopoietic tumors. Conversely, these neoplastic cases suggest the existence of two normal NK subpopulations that (*a*) are distinguishable by the presence of Tβ gene rearrangements and T3 antigen expression, and (*b*) express T8 and Mol antigens.

The existence of two LGL/NK subpopulations can be explained by at least two possibilities. First, they may reflect two separate differentiation lineages. For example, $T3^+T\beta^+$ NK clones and tumors may represent proliferations of peripheral T cells displaying NK-like phenotype, morphology, and functions, while $T3^-T\beta^-$ cells may belong to a non–T cell-related lineage. Alternatively, a common differentiation pathway may exist in which, together with the appearance (or disappearance) of various lymphoid or myelomonocytic markers (e.g., T8 and Mol antigen), LGL/NK cells can acquire the ability to express the T3/T cell antigen receptor complex. This latter possibility is supported by recent studies from our laboratories (Pelicci, manuscript submitted for publication) showing that peripheral blood LGL/NK cells do not express the T3 antigen, do contain a germline Tβ gene, and do not express Tβ or Tα mRNA. However, Tβ gene rearrangements and expression, accompanied by Tα gene and T3 antigen expression, can be induced in vitro in a $T8^+$ LGL/NK cell subset upon cultivation with IL-2. These findings suggest the existence of an IL-2-dependent LGL/NK differentiation pathway that appears to be clearly distinct from the conventionally defined T cell lineage, based on a number of features including: (*a*) its thymus independence, (*b*) the expression of specific LGL markers, and (*c*) the coexpression of T8 and Mol antigens. We propose that LGL isolated from patients with Tγ LPD may represent expansions of cells blocked at different stages of differentiation/activation within the LGL/NK hematopoietic lineage(s).

The often chronic, noninvasive clinical behavior of Tγ LPD has led to the hypothesis that it represents a reactive rather than a neoplastic process (20). However, our studies demonstrate that the cellular expansion is monoclonal in origin, at least in the Tγ LPD cases displaying Tβ gene rearrangements. It is intriguing that the majority of cases of Tγ LPD are $T\beta^+T3^+$ when the majority of peripheral blood normal LGL/NK cells are $T\beta^-T^{3-}$. The latter finding

suggests that a presumably more differentiated/activated target is frequently involved in Tγ LPD.

Summary

Twelve cases of Tγ LPD (lymphoproliferative disorders of Fcγ receptor–bearing T cells) involving an expansion of large granular lymphocyte/natural killer (LGL/NK) cells were investigated for the expression of LGL/NK-associated markers and for Tβ gene rearrangement. All the cases selected were classified as Tγ LPD on the basis of morphology, function, and phenotype of the circulating cells. 10 to 12 cases displayed clonal rearrangements of the Tβ locus and expression of the T3 antigen, whereas the 2 remaining cases displayed the germline configuration of the Tβ gene and no expression of the T3 antigen. T8, Mol, B73.1, and N901 antigens were variably expressed among both $T\beta^+T3^+$ and $T\beta^-T3^-$ Tγ LPD cases. We suggest that individual Tγ LPD cases represent the clonal expansion of cells frozen at different stages of differentiation/activation within an individual hematopoietic LGL/NK lineage.

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