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Molecular Analysis of TCR Clonotypes in LGL: A Clonal Model for Polyclonal Responses

Christine L. O’Keefe,* Magdalena Plasilova,* Marcin Wlodarski,* Antonio M. Risitano‡, Alexander R. Rodriguez,* Evan Howe,* Neal S. Young‡, Eric Hsi‡, and Jaroslaw P. Maciejewski2*

Large granular lymphocytic (LGL) leukemia is a clonal lymphoproliferative disorder of CTL associated with cytopenias resulting from an immune and cytokine attack on hemopoietic progenitor cells. Extreme clonality of CTL expansions seen in LGL leukemia makes it an ideal model to study the role of the T cell repertoire in other less-polarized immune-mediated disorders. Complementarity-determining region 3 (CDR3) of the TCR is a unique Ag-specific region that can serve as a molecular marker, or clonotype, of the disease-specific T cells. We studied the variable portion of the β-chain spectrum in a cohort of LGL leukemia patients. The CDR3 sequences were determined for the immunodominant clones and used to design clonotype-specific primers. By direct and semi-nested amplification, clonotype amplicons were found to be shared by multiple patients and controls. Analysis of the generated sequences demonstrated that the original clonotypes are rarely encountered in normal control samples; however, high levels of homology were found in both controls and patients. Clonotypes derived from individual LGL patients can be used as tumor markers for the malignant clone. More generally, clonotypic analysis and comparison of the variable portion of the β-chain CDR3-specific sequences from a large number of patients may lead to better subclassification of not only LGL but also other immune-mediated disorders. The Journal of Immunology, 2004, 172: 1960–1969.

Large granular lymphocytic (LGL) leukemia is a clonal lymphoproliferative disorder of CTL (1) frequently accompanied by cytopenias (2–8). In contrast to typical lymphoid malignancies, the LGL clone does not appear to be entirely autonomous, and except for its monoclonal nature it resembles a stereotypical antigenic CTL response (6). Clearly, it is possible that LGL leukemia represents a disregulated reaction to a viral infection or self-Ag. Such a response could be, for example, maintained by a sustained antigenic drive or by a genetic insult to a specific T cell clone rendering it insensitive to physiologic apoptotic signals (9–11). It is possible that the inciting triggers and those that maintain continuous clonal expansion differ. The lineage restriction of hemopoietic inhibition in LGL suggests that clonally expanded CTL specifically recognize lineage-committed hemopoietic progenitor cells in the bone marrow, resulting in the characteristic cytopenias (2–4, 8, 12). Furthermore, frequent association of LGL expansion with rheumatoid arthritis (13–15) and Felty’s syndrome (15–17) suggests that self-triggers outside hemopoiesis may be involved in the context of different clinical features.

Oligoclonal expansions of T cells have been identified in a number of other immune-mediated conditions, including infections (e.g., EBV and CMV) (18–20) and graft-vs-host disease (GVHD) (21–23); but in contrast to LGL leukemia, these clonal expansions are less pronounced and routine TCR rearrangement studies are negative. In addition to the classical presentation of LGL leukemia with refractory anemia or neutropenia, LGL has also been found in association with myelodysplastic syndrome (MDS) (24, 25), aplastic anemia, (26) and paroxysmal nocturnal hemoglobinuria (PNH) (27). In all these conditions a T cell-mediated process has been implicated in the pathophysiology of hemopoietic inhibition, and oligoclonal CTL expansions have been described (2, 8, 25, 27, 28). Although typical autoimmune processes are polyclonal in nature, LGL leukemia may represent a suitable model to study changes and regulation of the CTL repertoire during disease.

TCR is a heterodimer, comprised of αβ or γδ chains, encoded by V(D)J segments and a constant region (29–31). Somatic rearrangements between the V, D, and J regions give the TCR both its heterogeneity and its fine specificity for Ags. The complementarity-determining region 3 (CDR3) of the β-chain (VB) of the TCR is the structure recognizing antigenic peptides in the context of HLA, and the sequence of this unique Ag-specific region can act as a molecular marker, or clonotype, of the disease-specific T cells (32). During rearrangement, transferases add or remove nucleotides at the Vβ-Dβ and Dβ-Jβ junctions, resulting in CDR3 regions that can vary in length by several amino acids. This additional level of heterogeneity ensures that an almost unlimited pool of sequences can be found within VB CDR3 regions. In addition, these clonotypes can serve as surrogate markers for the unknown Ags driving the T cell response.

We have applied a molecular TCR repertoire analysis to identify and characterize LGL-specific clonotypes in a large cohort of patients with LGL leukemia and to study their utilization patterns in LGL leukemia patients and healthy controls.

Materials and Methods

Patients and controls

We obtained samples from a total of 33 patients with LGL leukemia and 24 healthy controls. Informed consent for sample collection was given by the
individuals according to protocols approved by the Institutional Review Board of the Cleveland Clinic Foundation (Cleveland, OH) and the National Heart, Lung, and Blood Institute of the National Institutes of Health (Bethesda, MD). The original diagnosis of LGL was established by modified clinical and laboratory parameters as suggested by Semenzato and colleagues (33) but we also included cases that do not meet the current clinical criteria for LGL, but which have an expansion in a specific T cell population.

MDS diagnosis when applicable was established by marrow biopsy and peripheral blood counts criteria and classified according to Fab classification (34) and based on parameters specified by the International Scoring System for MDS (35). The presence of a PNH clone was determined by flow cytometry; the test was considered positive when >1% of GPI-anchored protein-deficient neutrophils in blood were found as defined by negativity for surface staining for CD66b and CD16 in a distinctive population of CD15+ cells (in our cohort, only one patient showed a coinciding PNH clone).

VB cytometry (VB “spectratyping”)

Fresh peripheral blood (PB) was stained for VB flow cytometry analysis according to the manufacturer’s instructions (IOTest Beta Mark kit; Beckman Coulter, Fullerton, CA), with the following modifications: 5 µl PC5-conjugated CD4 and 5 µl of ECD-conjugated CD8 mAbs were added to each tube to quantitate the contribution of each VB family to the CD4 and CD8 lymphocyte pools. A four-color protocol was used. The lymphocyte gate was set according to their size and forward scatter properties (36). VB family usage was determined within the CD4 and CD8 lymphocyte fractions using the appropriate gates. Initially, nine normal samples were characterized to define the average size and SDs for the VB repertoire as defined based on its mean ± 2 SD over the average VB family size. As the Ab set contained in the IO Beta Mark kit does not cover the whole VB spectrum, four patients were identified who did not have expanded clones by flow cytometric VB analysis. Amplification products for VB families 6, 15, and 24 were obtained by VB PCR and CDR3 size distribution analysis was used to determine the oligoclonally skewed VB family (see below).

Lymphocyte separation

Mononuclear cells were separated from total PB by density gradient sedimentation (Mediatech, Herndon, VA). CD4+ and CD8+ T cells were isolated by positive and negative selection from PB mononuclear cells using a commercially available magnetic bead separation system (MACS; Miltenyi Biotech, Auburn, CA) according to the manufacturer’s instructions. Alternatively, samples were stained with a CD5 mAb conjugated with FITC (BD PharMingen, San Diego, CA) and sorted on an Epics Altra high-speed flow cytometer (Beckman Coulter).

RNA isolation and cDNA synthesis

Total RNA was extracted from CD8+ T cells with TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was generated by first strand cDNA synthesis using the SuperScript II RT Kit (Invitrogen).

CDR3 region amplification

cDNA was amplified using PCR with 22 different VB family-specific primers and a constant region primer (CB) (30, 31). VB10 and VB19 are pseudogenes and therefore were not included. Four microliters of TaqMaster PCR Enhancer (Eppendorf, Hamburg, Germany), 2 µl of 10× buffer containing 15 mM magnesium acetate, 0.4 µl of 10 mM dNTP (10 mM each dGTP, dATP, dTTP, dCTP), 1 µl of 20 µM CB primer, 2 µl of CDNA, 5 µl of 4 µM VB family primer, and 0.2 µl of Taq (5 U/µl, Eppendorf) were mixed in a final volume of 20 µl. For cloning, an unlabelled CB primer was used. PCR was performed in a Mastercycler thermocycler (Eppendorf) under the following conditions: 15 cycles of touchdown PCR (denaturation at 95°C for 1 min, annealing at an initial temperature of 60°C for 1 min with a -0.5°C reduction of temperature for the subsequent 14 cycles to a final annealing temperature of 53°C and extension at 72°C for 1 min) was followed by an additional 20 amplification cycles (95°C for 1 min, 53°C for 1 min, 72°C for 1 min). A final extension was performed at 72°C for 10 min.

CDR3 size analysis (CDR3 “spectratyping”)

One microliter of amplification products generated with a fluorescently labeled CB primer was mixed with 12 µl of Hi-Di formamide (Applied Biosystems, Foster City, CA) and 0.5 µl of GeneScan 400HD ROX size standard (PErkinElmer, Foster City, CA). The samples were run on an ABI Prism 310 Genetic Analyzer (Applied Biosystems) and analyzed using Ge-
28 to 80 (median age, 59.1 years). Neutropenia was the most common presentation (17 of 33), while in 8 patients anemia was found. In a further 5 patients, a bi-lineage cytopenia (neutropenia/anemia or anemia/thrombocytopenia) was present; 3 patients showed pan-cytopenia. Four of the patients had a concomitant diagnosis of MDS (2 refractory anemia, 2 refractory anemia with excess of blasts); in one patient a significant PNH clone was present. Additionally, 4 patients had a history of autoimmune diseases: patient 12 had rheumatoid arthritis, patients 15 and 18 had ulcerative colitis, and patient 8 had multiple sclerosis.

**VB utilization pattern in LGL**

In our initial studies we determined the VB utilization pattern of the LGL CTLs using flow cytometry and a set of VB-specific mAbs (Table I). The panel of anti-VB Abs used here covers ~70% of the total TCR VB spectrum. Normal values have been determined for healthy controls (n = 9), and were found comparable to the previously published values (n = 36) (37). For the purpose of this study, we defined a significant expansion as one that was greater than the mean + 2 SD. The relative contribution of the expanded T cell clone to the total CD8⁺ T cell pool varied between patients, from 7 to 93% (mean 49.2%, SD 27.8%, Table I). The Ab panel detected at least one significantly expanded VB family in all patients but four (6, 15, 29, and 32) in whom the sum of all VB fractions was significantly lower than the expected value of 65%, suggesting that the VB family containing the LGL clone was not detected by the available Abs. For these patients, products for the VB families not covered by the Ab panel (VB6, 15, and 24) were amplified in a PCR using VB family-specific primers and spectratyped to determine monoclonality. Additionally, we were unable to get VB Ab staining for patient 8, despite using several separate samples. All VB family-specific PCR products were amplified from the sample and spectratyped, and the immunodominant clone identified by its monoclonality.

Four patients had expanded clones in two VB families: 7 (VB23 and 14), 15 (VB24 and 6), 17 (VB3 and 21), and 18 (VB17 and 3). No VB family was significantly overrepresented among the expanded T cell clones, and those families detected occurred in ~2–13% of LGL clones studied (Fig. 1A). Four VB families (VB4, 5, 11, and 16) were not used by the expanded LGL clones in this cohort of patients.

**TCR utilization in normal controls**

To compare the degree of the clonal expansion in LGL with the clonotypic variability under normal conditions, we characterized
utilization patterns in normal control samples (n = 10), of which five were studied for VB3 and VB13 respectively. An average of 36.2 clones per VB family per normal control was sequenced. In all 10 samples, at least one T cell clone was found to be represented by two or more identical sequences in the total sequence pool. We have defined the percent redundancy as N clones with identical sequences/N clones sequenced (Fig. 2). The redundant clones contributed 1–21.4% of the VB family repertoire (mean 6.4%, SD 4.8%). However, only 2 of the 37 redundant clones found in normals were significantly expanded (NC1/VB3, 21.4%; NC7/VB13; 18.6%). In contrast, for LGL CTL clones the redundant clones contributed 22–100% of the total sequences within a given VB family (mean 87.4%, SD 23.5%), and all 28 clones were significantly expanded.

**Molecular analysis of TCR utilization pattern**

An expansion of an entire VB family may indicate the presence of clonal lymphoproliferation but may be also due to the effect of superantigens. To enrich for CTL, we purified CD8\(^+\) T cells. PCR primers, specific for the expanded VB families, were used to generate CDR3-derived amplification products (clonotypes). Either the PCR products were directly sequenced, or products were gel-purified and ligated into a TA vector and a large number of clones sequenced.

**FIGURE 1.** VB and JB region utilization in LGL CTL clones. A, VB utilization by the expanded T cell clones of the original cohort of 33 patients is shown. Expansions are expressed as a percentage of the total number of expanded T cell clones (because several patients had two expanded clones, the number of expanded clones is greater than the number of patients). Four VB chains (VB4, 5, 11, and 16) were not found within the expanded clones in this cohort of patients. B, JB utilization within the cohort is shown as a percentage of the total number of expansions (also greater than the number of patients).

**FIGURE 2.** Frequency of immunodominant clones within expanded VB families. The contribution of each expanded CTL clone to the VB family spectrum for 26 patients (P1 through P25 and P30), as well as expansions normally occurring within VB13 and VB3 in 10 healthy controls (NC1 through NC5 for VB13, and NC6 through NC10 for VB3) are shown. For comparison, expanded VB13 families (patients and normal controls) are shown in gray, while samples expanded for VB3 are shown in white.
Table II. Clonotypes of LGL leukemia expanded T cell clones

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*CDR3 sequences are given in amino acid code. The first invariant amino acid of the J region is indicated. Percentage of identical sequences within a given VB family.

Of the original 33 patients, TCR utilization analysis was performed on 26 patients; 28 LGL-specific clonotypes were identified (Table II). Two joining regions, J1.3 and J2.2, were not used by the expanded clones (Fig. 1B). In contrast, joining regions J2.1 and J2.7 accounted for ~50% of J regions used by LGL clones. All 28 TCR NDN regions had different nucleotide and amino acid sequences. We compared the VB/NDN/J sequences among all patients and within subgroups, including VB family, JB, type of cytopenia, and the presence of HLA-A2. No significant sequence similarity was detected between the clones in the groups tested using the ClustalW protein alignment algorithm (www.ebi.ac.uk/ clustalw/index.html). Several patients had two expanded clones, and comparisons of the clones within each patient also showed no similarity. Four VB3 and two VB13 LGL CTL clones were compared with respective sequences from multiple normals and no significant homology was found.

We then compared the LGL-specific clonotypes to a database containing 4393 clonotypes (2036 normal control sequences, 380 sequences from bone marrow failure disorders and LGL leukemia, and 1977 derived from other immune-mediated conditions), including those that contain sequences generated in our lab as well as those that have been published or deposited in GenBank. A 6-substitution comparison (see Materials and Methods) identified one shared clonotype, PBBBBBBIJZ, between patient 18 and a clonotype derived from a muscle biopsy from a patient with inclusion body myositis (38). The amino acid sequence for the clonotype from patient 18 was PLGAVGYN and PGLAGIYN for the published clonotype. Although the VB family used by the clonotypes differed (VB3 vs VB5), the clonotypes shared a common joining region, J2.1. Several patients (for example, patients 1 and 15, see Table II) had minor clones that differed in their NDN sequences from the major immunodominant clone. Sequence similarities between several of these minor clones and database clonotypes were also identified (data not shown).

Clonotypic PCR analysis of patient and normal samples

The LGL clonotypes can be informative in a number of ways. They are unique markers for the individual patient’s disease, and since these sequences reside at the unique-specificity Ag recognition site of the TCR, they can be used as surrogate markers for the target Ags. The frequency of these clonotypes among LGL leukemia patients and healthy controls may give some indication as to the nature of the Ags. The presence of the same clonotype in numerous patients may suggest that all are responding to a broadly distributed Ag, with a pathogenic role in the disease. Restriction of a clonotype to a few patients may reflect differences in the immune response related to HLA-restriction or resulting from a response to more singular Ags, resulting from viral infection or neo-expression. Conversely, if the same clonotype is present in numerous patients and healthy controls, it would suggest that the clone is responsive to a very common and immunodominant Ag, likely infection-related. However, even in this latter case we cannot exclude that the triggering Ags may be autoantigens, reflecting escape from immune surveillance. Titer and phenotypic features of the clone should explain the nonpathogenic role in normals.

To efficiently determine the uniqueness of LGL-associated clonotypes in a large number of individuals, we designed 11 PCR primers from the LGL CTL clonotypic (unique NDN) sequences, as well as from three previously published clonotypic LGL sequences (25) (see Materials and Methods for primer sequences). When used in a direct PCR assay, all primers amplified products from the original patient samples from which they were derived (Fig. 3A). Amplification results from healthy control samples differed for individual clonotypes. Four primers (primers 2, 3, 4, and 18) created strong products only from the patient samples, while four others (primers 12, 14, 34, and 35) amplified from one normal (N6). Primer 7 produced products from samples N3 and N6; primers 5, 6, and 36 amplified from three controls (N4, N5, and N6), while primer 9 amplified products from five controls (N2, N3, N4, N5, and N6). Although the NDN region of TCR CDR3 plays an eminent role in Ag recognition, the HLA presentation in the context of a specific allele also has a large impact on immune response to Ags. Therefore, we tested primers derived from patients that were HLA-A2 against HLA-matched healthy controls (Table I, Fig. 3B). Primers 2, 3, and 4 amplified products from the patient sample only, while primer 12 amplified from three HLA-A2 normal samples.

We further analyzed the amplification products from one patient, N6, by CDR3 size analysis. Products from 3 primers (3, 12, and 18) were monoclonal, while products from 5 (4, 5, 14, 35, and 36) were polyclonal. Upon examination, primer 3 actually spanned the NDN/J junction, making the primer more specific for the clonotypic sequence. Sequence analysis of the oligoclonal products revealed the presence of the original clonotype only for primers 3 and 18. The oligoclonal product generated by primer 12 had the same NDN sequence, but used a different J region than the patient sequence. Polyclonal products contained sequences related to, but not identical with, the original patient clonotype.

To maximize our ability to detect rare sequences, we repeated the clonotype-specific utilization assay using a semi-nested PCR method. LGL leukemia patient and healthy control samples were
amplified with primers specific for the VB family of the expanded CTL clone and visualized by gel electrophoresis. Subsequently, amplified products were used as the template for the clonotype-specific primers. All primers amplified products from the original patient sample (Fig. 4A). The primer panel also amplified products from multiple LGL leukemia patients and control samples.

The surprising presence of amplification products from disease-derived clonotypes in healthy individuals as well as multiple patients led us to question the fidelity of semi-nested clonotypic PCR as a tool to detect specific clonotypes. Size analysis of semi-nested clonotype-derived products generated in control samples demonstrated polyclonality, while products from the original patient samples were monoclonal (Fig. 4C, data not shown). Subsequent cloning and sequencing of these PCR products verified the spectratyping data. The original clonotypic sequence was the only sequence detected in the patient samples, while similar but nonidentical sequences were amplified from normal samples. In contrast, patient clonotypic sequences were detected in normal control samples by direct clonotype-specific amplification (Fig. 3, A and C).

Effects of immunosuppressive therapy on LGL-specific expansions

A clonotypic sequence can be used to follow the expanded clone through the course of a disease, including relapse and treatment. Initially, we identified an expansion of VB17 for patient 3, comprising 83% of the entire T cell repertoire. Sequencing identified a clonotype that was present in 100% of the sequence pool, and a clonotypic primer was designed from this sequence. The patient was treated with oral cyclophosphamide for three months, and was found to be in complete remission by cell counts. A posttreatment sample was obtained and clonotypic analysis was repeated for VB17. VB17 was still oligoclonal by spectratyping. Although the original clonotype was not found, the sequences detected in remission were very similar (Table III). Direct PCR with the original clonotype-specific primer (primer 3) did produce an amplification product that was monoclonal by spectratyping and identical by sequencing. In a second patient studied serially, immunosuppression resulted in the decrease in the pathogenic clone. Similarly, the size of the affected VB family decreased posttreatment (not shown).

Discussion

In this report, we have identified and characterized LGL-specific CTL clones in a large cohort of patients with LGL leukemia. Although several reports have identified LGL-specific clonotypes in a variety of settings (17, 25, 39, 40), our study includes the largest number of patients and doubles the number of clonotypes reported. We found that no specific VB family was preferentially used by the LGL CTL, in agreement with earlier reports (41, 42). Another study (43) reported VB2 and 3 to be more frequently represented among LGL leukemias; however, these VB families appear to comprise a higher portion of the VB repertoire in normal samples, explaining their more frequent expansion in patients. Additionally, several VB families (VB4, 5, 11, 15, and 16) and JB regions (JB1.3 and 2.2) were not encountered in our cohort of LGL patients. Because of a limited sample size, these regions may not have been found due to random chance. If VB and JB utilization is entirely stochastic in LGL leukemia T cells, the frequencies at which each family is used would result from the pathogenic clone. Similarly, the sequence of the affected VB family decreased posttreatment (not shown).
would expect to find one expansion in this VB family. We derived normal values for JB region utilization from a large sequence database developed in our laboratory and found that like VB, JB utilization in LGL approximated that occurring in controls (except for JB 2.2). JB 2.2 is used in 8.91% of normal controls, suggesting that in our LGL group we should find JB2.2 clones. Therefore, we cannot exclude a nonrandom usage of VB and JB regions in LGL leukemia.

### Table III. Clonotype utilization pre- and posttreatment by sequence analysis

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pretreatment</th>
<th>Post-treatment/Remission</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NDN/J</td>
<td>JB</td>
</tr>
<tr>
<td></td>
<td>ASS</td>
<td>LPG1/1PE 1.1</td>
</tr>
</tbody>
</table>

A

![Figure 4A](http://www.jimmunol.org/)

B

![Figure 4B](http://www.jimmunol.org/)

C

![Figure 4C](http://www.jimmunol.org/)

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*Two LGL leukemia patients who were treated during the course of our study were analyzed in remission to determine clonotype utilization. Treatment consisted of oral cytoxan. Sequences detected are shown, with the NDN region indicated in bold. The J region is given in parentheses, and the percent contribution of each clonotype to the sequence pool is indicated.*

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indicating the presence of 2 immunodominant LGL clones. This finding is difficult to reconcile with the clonal nature of the malignant process for LGL leukemia, and suggests a strong similarity to natural polyclonal immune responses. In general, highly expanded VB families were more likely to contain only one clonotype (patients 2, 3, and 4), while those that were expanded to a lesser degree were not strictly monoclonal. Twenty-one of the 28 VB expansions characterized by sequencing were monoclonal, while 7 expansions were oligoclonal (Table II). The median level of expansion was 54% (SD 29.9%) for monoclonal families and 35.5% (SD 24.1%) for oligoclonal families. In 3 of the 7 oligoclonal expansions, another “minor” clonotype was also found to be significantly expanded as compared with controls. However, nominally 35.5% (SD 24.1%) for oligoclonal families. In 3 of the 7 oligoclonal expansions, another “minor” clonotype was also found to be significantly expanded as compared with controls. However, nominally these expansions were much less pronounced. It is possible that proliferation of the most autonomous/dominant clonotypes results in an over-representation of an entire VB family.

Our study differed from other reports in that we have identified a relatively high proportion of patients with anemia and MDS patients with an associated LGL clone. We believe that this difference may be due to our rigorous screening of patients with all cytopenias for the presence of an LGL clone. This finding may also indicate that the predominance of patients with neutropenia in previous cohorts may not reflect the true spectrum of hematologic abnormalities found in LGL leukemia.

We originally hypothesized that LGL-specific CTL clones were responding to Ags on hemopoietic progenitor cells, and therefore patients with similar cytopenias would have similar CDR3 structures. Our structural analyses detected no homology for these comparisons and others, including VB or JB utilized. The heterogeneity of clonotypes is not surprising given the extent of diversity of the HLA background. Nevertheless, several conclusions can be drawn from these findings. First, the number of patients we have studied may not be sufficient to detect such similarities. Second, we have not identified the context in which these patients may be subgrouped into distinct subsets. For example, LGL-specific clones may be HLA-B, and not HLA-A, restricted and consequently identification of different clonotypes may not preclude the presence of a common target Ag. Alternatively, more precise subcategorization of the distinct cytopenias may reveal structural similarities, but the framework principles for CDR3-peptide interactions are poorly understood. Finally, it is possible that each LGL-specific CTL clone may be responding to a unique Ag presented on all common target cells (e.g., erythroid or myeloid precursors).

That the CTL clone is a part of an initially polyclonal response can be concluded from the results of TCR analysis after remission. Clones identified after clonal eradication of LGL may have been diluted by the presence of the LGL clone but they can be detected after immunosuppression. Their similarity is striking and suggests recognition of related Ags.

Our observations in LGL leukemia must be viewed in the context of the normally occurring heterogeneity of the T cell repertoire. Under normal circumstances, expanded clonotypes are found infrequently in healthy controls, and the expansions are less dramatic than those seen in LGL leukemia patients. Additionally, these clonal expansions do not lead to overrepresentation of entire VB families, in contrast to clonal expansions in LGL leukemia. To define the pathologic significance, immunodominance within a VB family has to be “multiplied” by its expansion factor. The pathologic relevance of “normal” expansions seen in healthy individuals are unknown and currently only numerical and statistical parameters can be used to distinguish normal from pathologic expansions. It is likely that normally occurring clonal expansions may reflect subclinical processes and/or immune reactions to the environmental antigenic input. Moreover, the probability of encountering identical sequences between individuals, including between identical twins, is extremely low, suggesting an overwhelming adaptability of the immune response to epigenetic factors. The diversification of the clonotypic repertoire explains our inability to find identical clonotypes, given the number of patients and clonotypes analyzed.

In addition to the quantitative aspects of clonal expansion, the specificity of LGL-associated clonotypes may be of utmost importance for understanding not only LGL leukemia but also other bone marrow failure states. We used a clonotype-specific PCR assay in part to address the question of shared target Ags in LGL clones from different patients. If LGL-specific clones respond to unique Ags, then all clonotypes would be unique. In contrast, if “public” Ags were the cause of the lymphoproliferation in LGL leukemia, then corresponding clonotypes would be shared between both patients and healthy controls. If PCR using clonotype-specific primers do not amplify products in any but the original patient sample, the Ag is unique for that patient, or the CTL clones for that Ag have been deleted in all others. However, if clonotype-specific primers do amplify products from samples other than the patient sample, the Ag is more ubiquitous but CTL clones for the Ag are under immune control in all except for the patient.

Based on these assumptions, we have applied various clonotype-specific PCR methods to study the distribution of clonotypes found in LGL leukemia among other patients and healthy controls. Our study clearly demonstrates the general utility of clonotype-based diagnostic strategies. The findings suggest that utilization patterns vary between clonotypes, consistent with the presence of both “public” and private specificities in LGL leukemia. Clearly, the sensitivity of PCR plays a role in the detection of clonotypes, as demonstrated by the difference between amplification products for direct and semi-nested PCR methods. Conversely, primer design appears to be extremely important for the specific amplification of the target clonotype. In this pilot study, our primers were specific for the NDN region of CDR3 and spanned the V/NDN, but not the NDN-J junction, except for primer 3. This design was intended to increase the sensitivity, but not the specificity, of the amplification.

As demonstrated by the sequencing results, such primers amplify not only the target clonotype in the patient, but also clonotypes that varied from the target region by the addition or subtraction of several amino acids. In contrast, primer 3 did amplify the patient clonotype from a normal sample, further underscoring the importance of primer design.

Primers designed to span the NDN-J junction will result in a high fidelity of amplification and detection of only the originally targeted sequence. With such highly specific primers, clonotypic PCR can be used, for example, for the detection of residual LGL clones in patients successfully treated with immunosuppressive therapy. In our study, therapy resulted in a significant decrease or even disappearance of the pathogenic clone as determined by sequencing. However, clonal persistence has been confirmed by PCR, a finding consistent with a high relapse rate of LGL leukemia. Sequence analysis of the clonotypic spectrum demonstrated that suppression of the pathogenic clone might result in the appearance of similar clonotypes.

Using direct PCR, oligoclonal products were amplified from normals. Although most LGL-associated clonotypes are not shared by healthy controls, in two instances we did detect identical CDR3 sequences derived from immunodominant clones in blood from a control individual. In contrast, by increasing the sensitivity of the PCR assay through adding rounds of amplification (semi-nested...
PCR), we obtained polyclonal amplicons from more normal samples than by direct PCR, but failed to detect the original target clonotype by sequencing. There are two possible explanations for the findings, both of which occurred in our study. First, the primers were not specific enough for the clonotype. In these cases, the significance of the presence of similar but not identical sequences were not specified in the study. The second, more interesting scenario was encountered, in which oligoclonal products were found in normal samples. This finding could imply that the target Ag is ubiquitous, but CTL responsive to it are disregulated in LGL patients.

The observed heterogeneity of the utilization pattern of LGL-specific clonotypes may be a reflection of the fact that LGL represents an immune response, rather than random expansion of a mutated T cell clone. For example, the LGL expansion may reflect a clonal response to a persistent antigenic drive. In the setting of MDS, dysplastic clones could contain target Ags. In other instances, viral pathogens could provide the inciting trigger Ags but presence of a cross-reactive autoantigen would secure a further stimulus for the expansion of LGL clones. This is in clear contrast to non-Hodgkin’s B cell lymphomas, which show progressive and seemingly unstoppable expansion independent of antigenic drive. In addition, coexisting pathologies, such as cytopenias attributed to the specificity of the rearranged IgG chains, have been only rarely described. Nevertheless, the surprising specificity to certain hematopoietic targets in LGL provides a link between this disease entity and other polyclonal bone marrow failure disorder syndromes.

In summary, we describe here molecular evidence for CTL clonality in immune-mediated cytopenias using TCR clonotypes derived from a large cohort of patients. Our findings support the idea that LGL leukemia is an Ag-driven response similar to other immune-mediated conditions, and not a strictly autonomous malignant proliferation. The principle of clonotype-based immune diagnostics as applied here to study LGL responses may, in analogy to traditional serology, have a diagnostic utility. Similarly, identification of the structure of the CDR3 region may facilitate the development of Id-based vaccination strategies.

References