The Lymphoproliferative Disease of Granular Lymphocytes: Updated Criteria for Diagnosis

By Gianpietro Semenzato, Renato Zambello, Gordon Starkebaum, Kazuo Oshimi, and Thomas P. Loughran, Jr

The lymphoproliferative disease of granular lymphocytes (LDGL), also referred to as LGL leukemia, is a heterogeneous disorder, but is clinically, morphologically, and immunologically distinct. Although LDGL has recently been included in the revised classification of lymphomas as an independent clinical entity, no consensus exists on the criteria to establish the diagnosis. The aim of this report was to refine the parameters needed to make the diagnosis of LDGL. We studied 11 patients with chronic granular lymphocytosis selected from among 195 cases observed by our institutions from three different geographic areas (North America, Europe, and Asia). These cases did not meet the current criteria for inclusion in LDGL, since all patients had less than 2,000 GL/μL. However, in each of these patients, we found evidence for expansion of a discrete GL population. Clonal rearrangement of the T-cell receptor (TCR) β gene was found in peripheral blood mononuclear cells (PBMC) of all nine patients with CD3+ LDGL. Using recently generated monoclonal antibodies (MoAbs) against the TCR Vβ gene regions, we identified a unique TCR Vβ on GL from each of three patients studied. In two patients with CD3- LDGL, we also identified a restricted pattern of reactivity, by staining with MoAbs against p58 antigen found on normal natural killer (NK) cells. The clinical features of these 11 patients with relatively low absolute number of GL were similar to those reported previously for patients with greater than 2,000 GL/μL. These data demonstrate that newer techniques such as MoAbs against Vβ gene regions and p58 molecules and molecular analyses are useful to identify expansions of discrete GL proliferations. Demonstration of an expansion of a restricted GL subset is evidence for the diagnosis of LDGL, even in patients with a relatively low GL count. Our results also contribute to distinguishing between the end of normality and the beginning of pathology in the broad spectrum of GL lymphocytoses.

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THE LYMPHOPROLIFERATIVE disease of granular lymphocytes (LDGL) is a heterogenous disorder, but is clinically, morphologically, and immunologically distinct. It results from the chronic proliferation of granular lymphocytes (GL) that may have either a CD3+ or a CD3- phenotype.1-4 Clonal proliferations of these cells have also been termed T- and natural killer (NK)-LGL leukemia, respectively.1 This new entity is widely recognized as an independent disease, and it has recently been included in the revised classification of lymphomas.5-8

The clinical and laboratory features of LDGL have been extensively defined in the last few years.5-8 The evidence of a granular lymphocytosis greater than 2,000/μL lasting for more than 6 months is currently regarded as the criteria to define the disease.2-4 In the largest study defining the natural history of this disorder,9 all cases that did not meet these requisites were excluded. However, there is still no consensus on the correct criteria to establish the diagnosis; in fact, several LDGL patients have been reported with GL less than 2,000/μL.10-19 Furthermore, since the initial definition of the disorder,1-4 new tools have been provided to better characterize the expanded cells, such as cytogenetic and molecular analyses and new monoclonal antibodies (MoAbs) both against the V-gene regions of the T-cell receptor (TCR)20 and the molecules of the p58 family on NK cells.21 On the other hand, several reports claiming the presence of clonal populations in normal individuals12,22,23 raise the point of defining the limit between the end of normality and the beginning of pathology.

Given the inadequacy of parameters used to date, and in particular the variability of the minimum number of GL mandatory to define the disease, the aim of this report was to refine previous criteria2-4 for the diagnosis of LDGL. To this end, we studied 11 patients with chronic granular lymphocytosis selected from among 195 cases observed at our institutions from three different geographic areas (North America, Europe, and Asia). These cases did not meet the current criteria for inclusion in the LDGL category. However, applying newly available tools, we were able to show a discrete expansion of GL in each patient, thus establishing updated parameters to define the diagnosis of this disorder.

MATERIALS AND METHODS

Study populations. Among 195 patients with LDGL observed at our institutions (Padua University School of Medicine, Padua, Italy; University of Washington, Seattle, WA; Juntendo University School of Medicine, Tokyo, Japan; and State University of New York, Syracuse, NY), 11 cases (seven men and four women) with relatively low GL counts have been identified. Most of these cases had not been previously reported, since the patients did not meet the criteria for inclusion in the LDGL category9:2 in particular, their GL peripheral blood count did not reach the cut-off limit of 2,000/μL. However, clinical and laboratory features of four patients were included in the data base of a review of LGL leukemia.1 In fact, the absolute numbers of GL in cases under study were 1,200/μL, 1,500/μL, 1,850/μL, 680/μL, 900/μL, 1,500/μL, 1,500/μL, 1,700/μL, 1,830/μL, 2,000/μL.
CRITERIA FOR DIAGNOSIS OF LDGL

Clinical findings. Table 1 summarizes the clinical features of the 11 patients under study. The mean age was 59.5 ± 11.3 years. The mean duration of disease was 59 months (range, 12 to 115). Data in these cases have been compared with the clinical data obtained in the largest series of patients available in the literature,9 most of them observed at our institutions.

Recurrent bacterial infections were present in 6 of 11 patients, as a consequence of severe neutropenia in 4 of 11. In 7 of 11 patients, an associated disease was demonstrated, including autoimmune disorders (in particular, rheumatoid arthritis) in four patients and pure red blood cell (RBC) aplasia, HBV infection, and neoplasia (ovarian cancer) in three other cases. Splenomegaly was detected in 5 of 11 patients and fever and systemic B symptoms were present in one, while lymphadenopathy and skin involvement were present in none.

Hematologic data. Relevant findings are reported in Table 2. Examination of peripheral blood showed the presence of typical GL characterized by a pale cytoplasm and prominent azurophilic granules.

Anemia (<10 g/dL) was observed in 5 of 11 patients and neutropenia (<2,000/µL) occurred in all cases, with four patients presenting severe neutropenia (<500/µL); thrombocytopenia (<100,000/µL) was observed in one case.

In four cases, a significant bone marrow lymphocytosis (>20%) was detected. In three cases, the infiltration was

### Table 1. Clinical Features in LDGL Patients With a Low Number of GL (<2,000/µL) as Compared With Findings Detected in Cases With Typical Disorder

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients With Low GL Count (&lt;2,000/µL)</th>
<th>Typical LDGL Patients (&lt;2,000/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>%</td>
</tr>
<tr>
<td>Sex (female/male)</td>
<td>6/5</td>
<td>65/86</td>
</tr>
<tr>
<td>Associated diseases</td>
<td>8/11</td>
<td>72.7</td>
</tr>
<tr>
<td>Systemic B symptoms</td>
<td>1/11</td>
<td>9.0</td>
</tr>
<tr>
<td>Recurrent infections</td>
<td>6/11</td>
<td>54.5</td>
</tr>
<tr>
<td>Oral ulcers</td>
<td>1/11</td>
<td>9.0</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>5/11</td>
<td>45.4</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>1/11</td>
<td>9.0</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>1/11</td>
<td>9.0</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>0/11</td>
<td>0</td>
</tr>
<tr>
<td>Skin involvement</td>
<td>0/11</td>
<td>0</td>
</tr>
<tr>
<td>Therapy required</td>
<td>9/11</td>
<td>81.8</td>
</tr>
<tr>
<td>Response to therapy</td>
<td>0/9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5/9</td>
<td>55.5</td>
</tr>
</tbody>
</table>

1,720/µL, and 500/µL. Among the 195 LDGL patients, all those with GL and a low white blood cell (WBC) count were included in the current study. The normal range for peripheral blood GL counts is 223 ± 99 GL/µL.2

A 6-month follow-up evaluation was requested from all patients to assess whether the lymphocytosis was persistent or transient in nature. Survival was calculated as of January 31, 1996. Complete remission was defined as the complete recovery of blood cell counts, including the percentage and absolute numbers of GL and the complete disappearance of clonal GL population, as detected by Southern blot. Partial remission was defined as a recovery in blood counts greater than 50%, without complete normalization at the molecular level.

### Table 2. Laboratory Features in LDGL Patients With a Low Number of GL (<2,000/µL) as Compared With Findings Detected in Cases With Typical Disorder

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients With Low GL Count (&lt;2,000/µL)</th>
<th>Typical LDGL Patients (&lt;2,000/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>%</td>
</tr>
<tr>
<td>Hemoglobin (&lt;10 g/dL)</td>
<td>5/11</td>
<td>45.4</td>
</tr>
<tr>
<td>WBC (&gt;5,000/µL)</td>
<td>1/11</td>
<td>9.0</td>
</tr>
<tr>
<td>Severe neutropenia (&lt;500/µL)</td>
<td>4/11</td>
<td>36.3</td>
</tr>
<tr>
<td>Platelets (&lt;100,000/µL)</td>
<td>1/11</td>
<td>9.0</td>
</tr>
<tr>
<td>Bone marrow infiltration</td>
<td>4/7</td>
<td>57.1</td>
</tr>
<tr>
<td>Clonal TCR (CD3+ LDGL)</td>
<td>9/9</td>
<td>100</td>
</tr>
<tr>
<td>Clonal NK populations</td>
<td>25/11</td>
<td>22.7</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not detectable.

* The positivity for HTLV-I p19 as reported in the majority of patients is considered indeterminate reactivity and not a true-positive.
diffuse, while in one it was characterized by a focal pattern; in two cases, a myelodysplastic pattern was also observed.

**Serologic abnormalities.** Relevant findings are reported in Table 2. Increased lactate dehydrogenase (LDH) levels were detected in one of seven patients. Polyclonal hypergammaglobulinemia was detected in three patients and circulating immune complexes in two of five patients tested. Antinuclear antibodies were detected in 3 of 10 tested cases, while antineutrophil antibodies were found in all cases tested (three of three).

Although all four patients tested demonstrated the presence of anti-EBV antibodies (IgG antiviral capsid antigen [VCA], or IgG anti–Epstein Barr nuclear antigen [EBNA]), evidence of EBV DNA was detected using PCR in one of four cases. One patient was seropositive for HCV and one for HBV, and another patient had antibodies to HTLV-I gag protein p19. The presence of antibodies against p19 protein is considered an indeterminate reaction and is not classified as positive using conventional criteria. For the purpose of completeness, we included data from typical LDGL patients from other reports in Table 2.

**Surface marker analysis.** Table 3 summarizes the phenotypic pattern of patients under study. Nine of 11 patients were characterized by a proliferation of CD3− CD16− lymphocytes, most of them bearing the CD16+ antigen. The expression of CD16 was not investigated in all cases using recently produced MoAbs, such as KD1 or VD4, which are able to identify the totality of CD16 cells. For this reason, CD16 antigen expression is likely to be underestimated in the cases reported. PBMC from all patients tested expressed αβ TCR. The TCR repertoire was explored using specific anti-TCR Vβ region MoAb in three patients, with a dominant TCR Vβ region being expressed in each case (Vβ6, Vβ8, and Vβ13, respectively). Interestingly, CD57 was found on GL of all patients under study.

Two cases were characterized by the proliferation of CD3− NK-type GL; in one case, CD16+ lymphocytes also expressed CD56, as normal NK cells do. A restriction of the p58 family antigen expression was reported in both CD3− LDGL patients; in one case, all GL expressed only p58/EB6 antigen (78% of PBMC; 98% of CD16+ cells), and in the other case, proliferating GL were double-negative for p58/EB6 and p58/GL183 antigens (<1% positive cells).

**Assessment of clonality and cytogenetic analysis.** Clonal disease was demonstrated in all cases characterized by the CD3+ phenotype, as detected by TCR clonal bands on Southern blot hybridization (Table 2). Clonality of CD3+ GL cannot be evaluated by TCR Southern blot, since this method is not informative in CD3+ LDGL. Techniques using X-linked restriction fragment-length polymorphisms are applicable only in female subjects, but we had no chance to test our patients. For the purpose of completeness of CD3+ clonal analysis comparisons, data of typical LDGL patients from other sources are included in Table 2.

In the six cases tested (four CD3+ and two CD3−), a normal karyotype was detected.

**Therapy and follow-up.** In two cases, the clinical course was indolent; the patients are alive (103+ and 23+ months) without any therapy. Other patients were treated with different approaches, including prednisolone, methotrexate, chloroethylenes (2CdA), granulocyte colony-stimulating factor (G-CSF), hydroxyurea, or splenectomy. The indication for therapy was represented by recurrent infections due to severe neutropenia in seven cases, and to anemia and progressive disease in one case each. Although the indications for initiating treatment were consistent with those of typical LDGL patients, the requirement of intervention in patients with a low number of GL is higher (81.8% vs 29.8%). No patient achieved complete remission on therapy. Three patients died: one from infections (n = 2), myeloproliferative disorder (n = 1), acute myelogenous leukemia (n = 1), and progressive disease (n = 1). Both the myeloproliferative disorder and acute myelogenous leukemia occurred in CD3+ cases.

**DISCUSSION**

This study updates the criteria used to define LDGL. In contrast to the established definition of LDGL, we found that an absolute count greater than 2,000/μL is not essential for the diagnosis, provided that the expansion of a discrete GL population can be demonstrated. The clinical and laboratory findings of these patients with low GL counts were similar to those of typical LDGL patients. Severe neutropenia leading to recurrent bacterial infections was the most common indication for treatment in both groups of LDGL patients. However, there was a suggestion that patients with low GL counts required therapy more often than typical LDGL patients (Table 1).

One mandatory parameter needed to establish the diagnosis of LDGL is the demonstration of the expansion of a discrete cell population. This can be strongly suggested by finding a homogeneous pattern of reactivity with MoAbs. Among the reagents currently used to define GL (CD8, CD16, CD56, and CD57), the evaluation of CD16 positivity is essential. In fact, using appropriate MoAbs, virtually all CD3− GL are CD16+. Since the CD3−CD16− subset accounts for less than 5% of normal cells in healthy subjects, the increase of this subpopulation is highly suggestive of CD3− LDGL. Other reagents have also been proven ex-
tremely useful in recognizing discrete cell subsets, for example. MoAbs against TCR Vβ regions in CD3+ related LDGL,27,28 and against determinants of the p58 family,21,26 in patients with LDGL of the NK-cell type (CD3+). Indeed, in this study, in all patients tested (three belonging to the CD3+ LDGL and two to the CD3− LDGL group, Table 3), we demonstrated a homogeneous, restricted pattern of reactivity with anti-Vβ (Vβ6, Vβ8, and Vβ13) or anti-p58 (EB6 and GL183) MoAbs.

Although reactivity with discrete MoAbs suggests expansion of a particular GL population, it is important to prove clonality. For CD3− LDGL, rearrangement of TCR genes can be demonstrated. In all CD3− cases with low GL counts, a clonal rearrangement of TCR genes was detected by Southern blotting; in one of these, it was investigated and confirmed by TCR Vβ sequencing,19 an additional technique that is useful to investigate the rearrangements of TCR genes and can provide the final proof of clonal disease.33 In a separate study using specific MoAbs, PCR, and Southern blot analyses,25 we demonstrated that the subset recognized by the relevant MoAb and PCR was identical to that accounting for the extra band identified by Southern blot. For CD3− LDGL, it is more difficult to prove clonality because of the lack of clonal markers; these cells do not rearrange TCR α, β, γ, or δ genes. Although no unique cytogenetic abnormalities have been demonstrated in LDGL patients, chromosomal analysis could be used as a marker of clonality.6,8 However, this method is of limited use due to the difficulty of growing these cells in vitro.6 In both of the CD3− LDGL patients, cytogenetic analysis were normal. In female LDGL patients, the X-linked polymorphism gene analysis is potentially informative for clonal determination; such analyses were not performed in this study. Since we identified only two patients with low GL counts belonging to the CD3− phenotype, any conclusion regarding this subset must be considered preliminary. However, the clinical findings, the persistence of granular lymphocytosis, and the restricted pattern of reactivity with anti-p58 MoAbs suggest that these cases also result from a clonal process.

Some cases of LDGL have been reported that appear polyclonal.1,2,7,8 In these circumstances, we believe that the 6-month follow-up period is necessary to exclude transient reactive lymphocytoses. However, the 6-month follow-up criteria is not necessary when clonality can be established and in patients requiring treatment within 6 months from diagnosis for aggressive disease. It is also conceivable that a polyclonal GL proliferation might represent a preneoplastic condition, since some evidence suggests that the pathogenesis of LDGL is a multistep process.3,7 In such cases, the clonal granular expansion might evolve from the abnormal immunoregulation of a response to inciting antigens. Indeed, there is increasing evidence that viral infection may be involved in the pathogenesis of some LDGL.7,24,34 In this study, three of 11 patients reported (two CD3+ and one CD3−) showed evidence of viral infections, such as EBV, HBV, and/or HCV.

While clonality is central in the diagnosis and management of LDGL, clonality alone does not establish the diagnosis. In fact, clonal populations have been detected during autoimmune processes, including rheumatoid arthritis,22 and in bone marrow transplantation recipients.35 Clonal populations in these settings are likely to represent the epiphenomenon of an immunoregulatory disorder. More intriguing is the demonstration of expanded clones in healthy individuals that increase in prevalence with age.23 Furthermore, studies in twins and families suggest that the CD8 expansions in normal subjects might arise in response to an environmental exposure.22 A useful parameter for differentiating these clonally expanded populations is the presence in healthy subjects of CD8 lymphocytes without typical cytoplasmic granules.23 When sorted, virtually none of the normal CD3+CD8+CD16− clonally expanded cells have GL morphology (B.L. Kotzin, personal communication, 1996). More importantly, the TCR Vβ clonal dominance seen in LDGL patients should be clearly distinguished from the oligoclonal TCR Vβ pattern identified in otherwise normal subjects. Clones identified in healthy individuals appear to be relatively minor subpopulations detected only by PCR analyses using the length of the CDR3 of TCR Vβ, as compared with the clonal dominance of patients with LDGL, easily identified by Southern blot.

In conclusion, we found evidence for expansion of a restricted GL population in each of these patients with low GL counts. Therefore, the absolute number of GL is not critical for the diagnosis of LDGL. We emphasize that a multiparameter analysis including clinical, hematologic, immunologic, and molecular data should be used to assess the diagnosis of LDGL.

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