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# Large granular lymphocytosis

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Abstract

***Background and Objectives.*** **An increased number of granular lymphocytes (GL) has been reported in various clinical conditions and is currently interpreted as a reactive process to an underlying antigen stimulation. In recent years, a disease characterized by a definite increase in granular lymphocytes has been identified and recognized as lymphoproliferative disease of GL (LDGL). The aim of this study is to review the clinical, biological and pathogenetic mechanisms leading to this disorder.**

***Design and Methods.*** **Criteria for the diagnosis, immunologic and molecular evaluation, clinical features and new therapeutic approaches are reviewed.**

***Results.*** **More than 500 patients have been adequately reported in the literature. Immunologic classification of this disease distinguishes a CD3$^+$ form which is more common, and a CD3$^-$ variant; this latter accounting for nearly 15% of LDGL cases. CD3$^+$ LDGL is symptomatic in approximately 50% of cases, neutropenia, infections and anemia being the most frequent findings. Clonality of the T-cell receptor is usually documented in these patients. Cytokines such as IL-2, IL-12 and IL-15 have been claimed to play a role in this disorder. Symptomatic patients may benefit from combination therapy with low dose methotrexate and steroids. CD3$^-$ LDGL are usually associated with viral infection of GL: in particular, Epstein Barr and human T lymphotropic virus I/II have been claimed to play a role. Neutropenia is usually less pronounced than in CD3$^+$ LDGL patients. Clonality has rarely been demonstrated; however, when present, it correlates with an aggressive clinical course. Spontaneous regression of lymphocytosis has been reported in both CD3$^+$ and CD3$^-$ patients.**

***Interpretation and Conclusions.*** **Lymphoproliferative disease of granular lymphocytes is a well recognized disorder which encompasses a large spectrum of conditions, ranging from mild asymptomatic lymphocytosis to aggressive, usually fatal, disorders. Diagnosis of this disease is related to the demonstration that a discrete subset of GL is chronically expanded. Therapy should be delayed in asymptomatic patients; however, when needed, the combination of methotrexate or cytoxan and steroids represents the best approach.**


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Large granular lymphocytes (LGL) represent a morphologically recognizable lymphoid subset accounting for less than 15% of the normal peripheral blood cell population. The large majority of these cells belong to the natural killer subset, characterized by the CD3$^-$ CD16$^+$ CD56$^+$ phenotype, the ability to display non-major histocompatibility complex cytotoxicity and a germ line configuration of the T cell receptor (TCR).[1] A small portion of peripheral blood LGL, usually less than 3% in healthy subjects, express the CD3$^+$ CD16$^+$ phenotype. These cells rearrange the T cell receptor, mediate non MHC-restricted cytotoxicity *in vitro* and are thought to represent *in vivo*-activated cytotoxic T lymphocytes (CTL).[2]

A disorder characterized by a definite and persistent increase in GL was first reported in 1977 by McKenna *et al.*[3] A critical question was to define whether these cell proliferations represented a reactive or a neoplastic condition. Proliferating GL in patients with LGL proliferation largely belong to the CD3$^+$ CD16$^+$ T cell subset (85% of all cases), usually expressing T cell receptor (TCR) monoclonal rearrangements.[4-7] The cases sustained by CD3$^-$ NK cells are less frequent (15%) and are mostly represented by polyclonal proliferations.[4-8] It is now well established that GL proliferations represent a heterogeneous but clinically, morphologically and immunologically distinct disorder. Clonal disorders of GL have recently been included in the *Revised European and American Lymphoma* (REAL) classification as a distinct clinical entity, classified under peripheral T-cell and NK-cell neoplasms.[9] The relevant findings characterizing LDGL patients can be summarized as follows: low to moderate lymphocytosis (usually below 20,000 cell/µL) sustained by GL; chronic, usually indolent clinical course; presence of neutropenia and/or anemia; association with chronic diseases, such as rheumatoid arthritis, chronic viral infections or neoplasms.

### *Diagnostic criteria*

The evidence of a granular lymphocytosis greater than 2,000/µL lasting for more than 6 months has been historically regarded as the most powerful criterion for the diagnosis of disease.[4-6] However, several reports described patients characterized by less than 2,000 GL/µL, increasing the confusion concerning the correct criteria for establishing the diagnosis. Data from the *Yorkshire Leukemia Group* recently indicated that chronic GL lymphocytoses are considerably more common than suggested in the literature.[10] These authors studied 870 different adult blood samples (primarily from patients with non hematologic disorders), considering a cut-off limit for patient inclusion >25% and/or $>1\times10^9$/L of morphologically defined GL and/or phenotypically-defined NK-associated cells. They showed that 31% of these cases had an increased proportion of GL and, in the majority of patients studied, this abnormality was still present six months later. In a retrospective review of 1,501 lymphoid flow cytometric samples of peripheral blood, a substantial quantitative increase in natural killer cells was observed in patients with lymphoma, leukemia, immune thrombocytopenic purpura, and myelodysplastic syndromes, although no data on follow-up were provided.[11]

Since the initial definition of this disorder, recently available new tools, including molecular analysis and new monoclonal antibodies (MoAbs), capable of characterizing the expanded cells better, have identified different subsets of proliferating GL. Concerning the T cell compartment, the generation of specific MoAbs recognizing conserved segments of Vα and Vβ regions of the TCR and the demonstration that GL identified with anti-TCR V region MoAbs and the clonal population recognized by Southern blot are identical, has provided a useful screening approach to identifying patients with LDGL.[12] Concerning the NK cell compartment, the definition of a series of new molecules involved in the functional activity of NK cells, in particular members of the p58 family, has expanded our knowledge of the NK cell repertoire and NK cell subsets.[13,14]

On the basis of the above quoted advances in the field, the criteria for diagnosis of LDGL have been updated and published in a recent paper.[15] An absolute GL number greater than 2,000/µL is no longer mandatory for the diagnosis, provided that the expansion of a discrete GL population can be demonstrated (Table 1). This can be strongly suggested by finding a homogeneous pattern of reactivity with the above quoted MoAbs or by molecular analysis by Southern blot or PCR which can recognize a monoclonal population. Since monoclonal T cell populations have recently been reported also in normal elderly individuals,[16] particular attention is recommended. It is worth mentioning that studies on families and identical twins have indicated that a clonal $CD8^+$ population may represent a response to environmental stimuli:[17] in these cases the lymphocytosis is usually characterized by $CD3^+$ $CD8^+$ $CD16^+$ lymphocytes without typical cytoplasmic granules. While clones identified in healthy individuals appear

**Table 1. Diagnostic criteria for lymphoproliferative disease of granular lymphocytes.**

| | |
|---|---|
| • increase in granular lymphocytes* | (by morphology and/or immunophenotypic analysis) |
| • demonstration of clonality | (TCR rearrangement, chromoso mal abnormalities, anti-TCRVβ MoAbs, X linked restriction fragment-length polymorphisms) |
| • accumulation of discrete cell subsets according to the expression of p58 family molecules (CD158a, CD158b) | |

*Normal value: 200-400 GL/µL.*

to include relatively minor subpopulations, sometimes identified only by cloning the complementary-determining region (CDR)-3 of the TCR Vβ chain, we would like to emphasize that the clonal dominance of patients with LDGL is usually identified by Southern blot analysis.

These data further support the concept that a multiparameter analysis including clinical, hematologic, immunologic and molecular data should be used to make the diagnosis of LDGL.

### *Clinical features*

Although pediatric cases have occurred, the disease affects older people (mean 60 years). Usually, less than 10% of patients reported are below 40 years of age. The disease is asymptomatic in nearly 30% of cases, with lymphocytosis representing the only observed hematologic abnormality. More frequently, the disease is symptomatic with symptoms mostly related to neutropenia. Fever is often reported and is most commonly due to bacterial infections, ranging from oropharyngeal and skin infections to severe sepsis. Opportunistic infections are uncommon. Anemia is another relevant finding; substitutive therapy is required in nearly 15% of cases. B-related symptoms (fever, night sweats, weight loss) are observed in nearly 25% of cases. Physical examination reveals spleen enlargement in nearly 50% of cases, while hepatomegaly is less common. Lymphoadenopathy and skin involvement are rare, but of adverse prognostic significance. Bone marrow involvement is a common feature in LDGL patients.

A relevant aspect of LDGL is the frequent association with other diseases (Table 2). A strong association has been reported between rheumatoid arthritis (RA) and $CD3^+$ LDGL (approximately 25% of cases),[18] with a clinical picture that is reminiscent of Felty's syndrome (neutropenia, RA and splenomegaly).[19] It has been suggested that the prevalence of $CD3^+$ LDGL in Felty's syndrome is probably underestimated.[20] Recently, it has been reported that patients with LDGL

**Table 2. Conditions associated with the lymphoproliferative disease of granular lymphocytes.**

| | |
|---|---|
| Rheumatoid arthritis | 25% |
| Anemia | 20% |
| Chronic infections | 20% |
| Hairy cell leukemia | 5% |
| Solid tumors | <5% |
| MGUS/multiple myeloma | <5% |
| Idiopathic thrombocytopenic purpura | <5% |
| Hodgkin's and non Hodgkin's lymphomas | <5% |
| Thymoma | <5% |
| Neuropathy | <5% |
| Transplantation | <5% |

and RA have the same high frequency of DR4 haplotype as patients with Felty's syndrome (90 and 86%, respectively), suggesting a common immunogenetic background for the two conditions.[21] Patients with CD3⁻ LDGL rarely have associated RA, whereas they frequently have associated chronic viral infections (see below).[22] An association with other malignancies is also reported. Among these, hematologic conditions are the most represented group including monoclonal gammopathies, multiple myeloma, myelodysplastic syndromes, Hodgkin's and non Hodgkin's lymphomas,[11,23] although non-hematologic neoplasms have been reported in association with LDGL.[24] Expansion of $CD3^+$ $CD57^+$ lymphocytes has also been demonstrated after bone marrow transplantation, usually representing a transient phenomenon, possibly related to an activation process due to graft-vs-host disease or cytomegalovirus infection.[25] We recently described the persistence of the same clonal population in a bone marrow recipient and his donor for two years after BMT, pointing out that GL proliferation can be horizontally transmitted (manuscript submitted). Clonal $CD3^+$ LDGL can be observed after organ transplantation.[26]

Recent data demonstrate an increasing association of LDGL with peripheral neuropathy, in some cases with histologically documented intraneural and epineural GL invasion.[8,27] Another intriguing association is between LDGL and pulmonary hypertension, with documented infiltration of the lung by GL (manuscript in preparation).

### Hematologic features

The hallmark of the disease is the presence of a peripheral blood lymphocytosis with the characteristics of GL. The typical morphology of GL in a LDGL patient is shown in Figure 1. The normal LGL count ranges from 200 to 400 cells/μL. These cells are usually large (15-18 μ), with abundant cytoplasm containing the characteristic azurophilic granules, and a kidney-shaped or round nucleus. In some cases, granules can be difficult to detect, despite the typical membrane phenotype. Positivity for non-specific esterase and periodic Schiff acid has been reported.[28]

Neutropenia, sometimes severe (<500/μL), is frequently reported. Adult onset cyclic neutropenia has been reported in some patients with $CD3^+$ LDGL. The mechanism accounting for neutropenia has not been elucidated, and both a direct inhibitory effect on CFU-GM, and anti-neutrophil antibodies have been described in LDGL patients. The recent demonstration that GL express and release Fas ligand has postulated a role for this molecule in the pathogenetic mechanisms accounting for neutropenia.[29]

Normocytic or macrocytic anemia is present in nearly 40% of cases. Pure red cell aplasia has been reported with unusual frequence in Japan.[6] The accumulating data convincingly suggest that proliferating GL may inhibit erythroid progenitors (both CFU-E and BFU-E).[30] Abnormal production of interferon-γ and tumor necrosis factor has been claimed to play a role in developing pure red cell aplasia. In some cases Coombs' positive hemolytic anemia has been demonstrated.

Autoimmune thrombocytopenia has been associated with $CD3^+$ LDGL. Abnormalities of humoral immunity have frequently been shown. Polyclonal hypergammaglobulinemia (mostly IgG and IgA),[31] high titers of rheumatoid factor and antinuclear antibodies are commonly reported, particularly in $CD3^+$ LDGL. Monoclonal gammopathy of either IgG or IgM has been documented in some cases.

### Immunologic and molecular features

Based on surface membrane phenotype, patients' GL can belong either to the T cell lineage or NK cell lineage.[4-6] $CD3^+$ GL usually express the $TCR\alpha\beta^+$, $CD2^+$, $CD4^-$, $CD8^+$, $CD16^+$, $CD57^+$, $CD45RA^+$, $CD122^+$ (p75 IL-2R), $CD25^-$ (p55 IL-2R) phenotype. CD56 antigen is usually not expressed, but its presence has prognostic significance.[32] Some cases express CD4 antigen, with or without CD8. Rarely

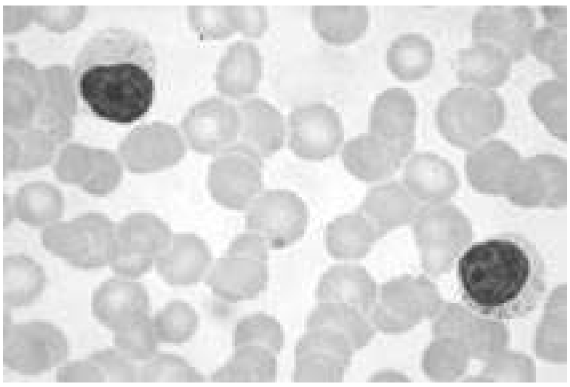

**Figure 1. Morphologic features of typical granular lymphocytes in a LDGL patient.**

the $CD4^-$ $CD8^-$ phenotype (either TCR$\alpha\beta$ or TCR$\gamma\delta$) has been reported. $CD3^-$ GL bear the $CD16^+$ $CD56^+$ $CD45RA^+$ $CD122^+$ $CD25^-$ phenotype. CD57 antigen is usually weakly expressed.

The clonal nature of $CD3^+$ GL is commonly assessed by Southern blot analysis of TCR $\beta$ and/or $\gamma$ genes or using specific primers for TCR V$\beta$ or V$\gamma$ by PCR. Most patients show a monoclonal rearrangement of TCR $\beta$ and $\gamma$ genes. Accordingly, PCR can easily show a dominant V$\beta$ region in the majority of patients. MoAbs recognizing different V$\beta$ regions of TCR have contributed to the in depth analysis of the TCR repertoire of patients. Using this approach we were able to demonstrate a preferential use of V$\beta$13.1 in 5 out of 18 patients studied.[12]

The clonal nature of $CD3^-$ GL, which do not rearrange TCR, is more difficult to prove. Studies on restriction fragment length polymorphisms and the methylation pattern of X-linked genes (PGK) have demonstrated the monoclonality of cell proliferation in some cases,[33] although the majority of cases are polyclonal.[34] Analysis of EBV genoma has also shown the clonal pattern of $CD3^-$ GL proliferation in patients with integration of EBV into GL.[35,36] The recent demonstration of a NK cell repertoire linked to the expression of molecules recognizing HLA-related antigens, and displaying inhibitory (*Killer Inhibitory Receptor*) or activating (*Killer Activatory Receptor*) signals,[37, 38] has led to investigation of whether the GL proliferations are sustained by NK subsets.[13] This approach is made possible by the stable expression of these antigens, which are not modulated by cell activation, proliferation or cloning. Of particular interest are the anti-p58 MoAbs CD158a (EB6) and CD158b (GL183), which distinguish four subsets of NK cells: $EB6^+$ $GL183^+$, $EB6^+$ $GL183^-$ $EB6^-$ $GL183^+$, $EB6^-$$GL183^-$. Using these antibodies in 14 LDGL cases we demonstrated that the majority of $CD3^-$ GL proliferations (11 out of 14 cases), are sustained by restricted populations, i.e. seven cases were double negative, three cases were $EB6^+$$GL183^-$ and one case was $EB6^-$ $GL183^+$. This finding indicates that, although polyclonal, the majority of NK cells in these patients are indeed limited to specific subsets.[14]

## Etiology

The etiology of LDGL still remains an enigma. Morphologic and functional features of proliferating GL indicate that they are *in vivo*-activated, antigen-driven cells. Sequence analysis of TCR in some patients has shown that GL are under antigenic pressure.[39,40] MoAbs to anti-CD3 (mimicking antigen activation), either alone or in association with cytokines, have been demonstrated to induce proliferation of GL.[41,42] In particular, it has been suggested that IL-2 (produced by T cells) is associated with the events inducing GL proliferation which is then maintained through an IL-15 (produced by monocytes) mediated mechanism.[43] On the basis of these observations, it has been postulated that LDGL may represent *in vivo* expansions of cytotoxic T lymphocytes (CTL) against unknown antigens. Great efforts have been made to identify putative pathogenetic antigens. The correlation between chronic viral infections and $CD3^-$ LDGL suggested a role for some viruses, namely EBV, HBV, HCV, HTLV I/II, in the pathogenesis of LDGL.[44] In some patients with EBV infection and LDGL, the virus has been demonstrated within GL of patients. Analysis of EBV-genoma termini has documented the presence of a single episomal form of viral DNA in GL, supporting a direct role for this virus in the pathogenesis of GL proliferation. The majority of cases are clustered in Japan and are characterized by a very aggressive behavior.[35] Interestingly, we recently demonstrated the spontaneous resolution of a $CD3^-$ LDGL in a patient with documented infection of GL by EBV, indicating that the virus by itself is not sufficient for maintaining the proliferation, thus a second event is needed.[41] Chronic persistent EBV infection of natural killer cells in patients with granular lymphocyte expansion has been previously reported by us and others.[45,46]

HTLV I/II has been claimed to have a role in the pathogenesis of some cases of LDGL, both $CD3^+$ and $CD3^-$. Antibodies to HTLV I/II have been demonstrated in the serum of LDGL patients, and HTLV I/II sequences have also been demonstrated in GL DNA in some patients.[47] It has recently been reported that serum from LDGL patients reacts with an envelope protein of HTLV I/II. Using the epitope mapping technique, the seroreactivity was detected at the specific BA21 epitope of this transmembrane envelope protein. It has been postulated that a protein with homology to BA21 may be related to the pathogenesis of LDGL.[48]

## Prognosis and therapy

Lymphoproliferative disease of GL appears to be a heterogeneous disorder presenting with a wide spectrum of different clinical features. In the largest published multicenter study including 151 cases, co-ordinated by our institution, mortality after 4 years of prospective follow-up was 20%.[49] Another recent study on 68 $CD3^+$ cases from a single institiution reported a survival of over 80% at 150 months.[7] The disease may run asymptomatic for many years in some patients, whereas in other cases therapy is needed, usually for cytopenia-related manifestations. The percentage of patients who require therapy at some time during the disease ranges from 30 to 70%, according to different series. Spontaneous disappearance of GL lymphocytosis has been reported, also in clonal cases.[50]

Since the usual clinical course of the disease is relatively favorable, the identification of features which predict poorer survival is critical. We found that a low number of GL, a low percentage of $CD57^+$ cells and fever at diagnosis were associated with a worse

**Table 3. Differential diagnosis of chronic granular lymphocytosis.**

| |
|---|
| CD3+ LDGL |
| CD3– LDGL |
| NK cell lymphoma |
| NK-like T cell lymphoma |
| $\gamma\delta$ T-cell lymphoma |
| Reactive GL proliferations occurring in patients with: |
| Solid tumors |
| Idiopathic thrombocytopenic purpura |
| Non Hodgkin's lymphoma |
| Hodgkin's lymphoma |
| Viral infections |
| Hemophagocytosis |

outcome.[49] Another study reported that lower absolute neutrophil counts and the presence of B-symptoms were associated with a lower probability of achieving complete remission.[7]

Standard therapy for this disease has not been defined. The most common indications for initiating therapy are symptomatic anemia, recurrent neutropenic infections or, more rarely, B-related symptoms. Immunosuppressive therapy with cytoxan and prednisone has been reported to be effective in obtaining durable remission.[7] The doses were 40 to 60 mg/day of prednisone and 25 to 100 mg/day of CTX. Following an initial response to therapy prednisone was tapered. This therapy obtained a response rate of 81% of patients treated and a molecular remission in nearly 20% of cases.[7] Another very interesting approach is the combination of low doses of oral methotrexate and prednisone, which has been reported to induce complete remission in nearly 50% of patients.[51] Methotrexate was administered orally as a low-dose pulsed therapy in split doses in the morning and evening, once weekly. Weekly doses were started at from 5.0 mg to 7.5 mg, with escalation up to 15 to 20 mg/wk (10 mg/m$^2$) over 1 to 3 months.[51] Alternative approaches including splenectomy, cyclosporin A, colony stimulating factors (both G-CSF and GM-CSF) and fludarabin have been reported to be effective in selected cases. Aggressive diseases have been treated with combination chemotherapy, usually with very poor results, consistent with the recently recognized T/NK cell lymphomas.[9] The demonstration that neoplastic cells express high concentrations of P170 glycoprotein (the product of multidrug resistance gene) may in part account for this feature.[52] In these cases, allogeneic bone marrow transplantation should be considered for young patients having a compatible donor.

## Conclusions

The lymphoproliferative disease of GL is characterized by well defined clinical features and laboratory data. The demonstration of clonality appears to be a crucial step in the diagnosis of disease, but it must be differentiated from clonal populations detected in autoimmune processes and in normal elderly individuals. By contrast, clonality is not detectable and lymphocytosis is self-limiting in many clinical conditions characterized by an increase in GL, including viral infections (EBV, HBV, HCV, HIV, CMV), idiopathic thrombocytopenic purpura, skin disorders and hemophagocytosis (Table 3). In conclusion, a multiparametric approach including clinical, hematologic, immunologic and molecular analyses is recommended for a proper definition of LDGL.

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*RZ and GS contributed equally to the conception and design of the paper, drafting the article and revising it critically, and both gave approval of the final version to be published.*

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### Disclosures

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