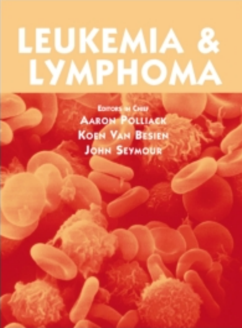

Leukemia & Lymphoma

ISSN: 1042-8194 (Print) 1029-2403 (Online) Journal homepage: http://www.tandfonline.com/loi/ilal20

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To cite this article: Cecilia M. Rodríguez, Carmen Stanganelli, Claudio Bussi, Daniela Arroyo, Darío Sastre, Viviana Heller, Pablo Iribarren & Irma Slavutsky (2017): Intracytoplasmic filamentous inclusions and IGHV rearrangements in a patient with chronic lymphocytic leukemia, Leukemia & Lymphoma, DOI: 10.1080/10428194.2017.1370549

To link to this article: http://dx.doi.org/10.1080/10428194.2017.1370549

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LEUKEMIA & LYMPHOMA, 2017
https://doi.org/10.1080/10428194.2017.1370549

LETTER TO THE EDITOR

Intracytoplasmic filamentous inclusions and *IGHV* rearrangements in a patient with chronic lymphocytic leukemia

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ARTICLE HISTORY Received 12 June 2017; revised 1 August 2017; accepted 15 August 2017

Chronic lymphocytic leukemia (CLL) represents the most common leukemia in the Western world, accounting for ~30–40% of all adult leukemias. The disease is characterized by a highly variable clinical course, ranging from indolent cases to patients with aggressive and rapidly progressing disease. Although staging systems are reliable predictors of outcome, they do not fully explain the heterogeneity in treatment response and survival. In the last decades, several prognostic biomarkers have been identified, allowing the subdivision of this heterogeneous disease into clinical relevant subgroups. Among them, genomic alterations and the *IGHV* (*immunoglobulin heavy chain variable region*) mutational status are of significance. Particularly, recurrent cytogenetic abnormalities namely deletions of chromosomes 11q, 13q and 17p and, trisomy 12, define subgroups of patients with different clinical behavior and response to treatment while *IGHV* mutational status permits to distinguish two major CLL subtypes, mutated (M) associated with a good prognosis, and unmutated (UM), characterized by a poor clinical evolution [1].

The presence of cytoplasmic inclusions of different structures is an uncommon event in lymphoproliferative and plasmacytic disorders. In CLL, the estimated incidence of crystals formation varies between 3 and 18% of cases [2,3]. These inclusions can present multiple morphologies including vacuoles, crystals, and pseudocrystals, and they are mainly localized within the rough endoplasmic reticulum (RER) compartment. Besides, most of the reported cases indicate that these inclusions represent immunoglobulin (Ig) deposits and usually the surface Ig, when it is demonstrable, is identical to the one found in the inclusion bodies [3–6]. However, the mechanism related to the inclusions formation remains to be determined.

In this article, we report a case of a 68-year-old woman referred to our Hospital on January 2003. At that time, routine analysis showed a total white blood cell count of 25.6×10^9/L (normal range: $4.5–10 \times 10^9$/L), 87% lymphocytes (normal range: 20–45%), with no anemia, thrombocytopenia or organomegaly. May-Grünwald-Giemsa (MGG) stained peripheral blood smear examination revealed mature lymphocytosis with moderate-sized lymphocytes, round nucleus, medium clumped chromatin and moderate cytoplasm with crystal inclusions, seen as rectangular unstained structures, in around 50% of total lymphocytes (Figure 1(A)). No bone marrow examination was performed. Serum protein electrophoresis and immunoelectrophoresis had a normal pattern. Lactate dehydrogenase (LDH) and β_2-microglobulin (β_2M) values were: 431 UI/L and 2.4 mg/L, respectively (reference values: LDH: 180–450 UI/L; β_2M: 0.8–2.20 mg/L). Flow cytometry analysis revealed a clonal B cell population with atypical CLL phenotype. B cells were positive for CD19, CD20 (dim), CD5, CD200, CD22 (dim), CD79b (dim), IgM, lambda light chain restriction (dim), but negative for CD23, CD10 and FMC7. Prognostic markers analysis showed 63% ZAP-70 positive cells; while CD38 and CD49d were negative. Thus, a diagnostic of CLL, Rai stage 0, without treatment requirement was done.

After nine years of follow up, the disease progressed to Rai stage III, with anemia, organomegaly and weight loss. As previously described, the MGG blood smears showed intracytoplasmic inclusions in 60% of the lymphocyte population. No significant changes on LDH and β_2M values were observed. At that moment, flow cytometry analysis showed two B cell populations, one of them similar to the first study and the other (5% of B cells) exhibiting higher lambda surface expression. In addition, the analysis of prognostic markers showed 74%

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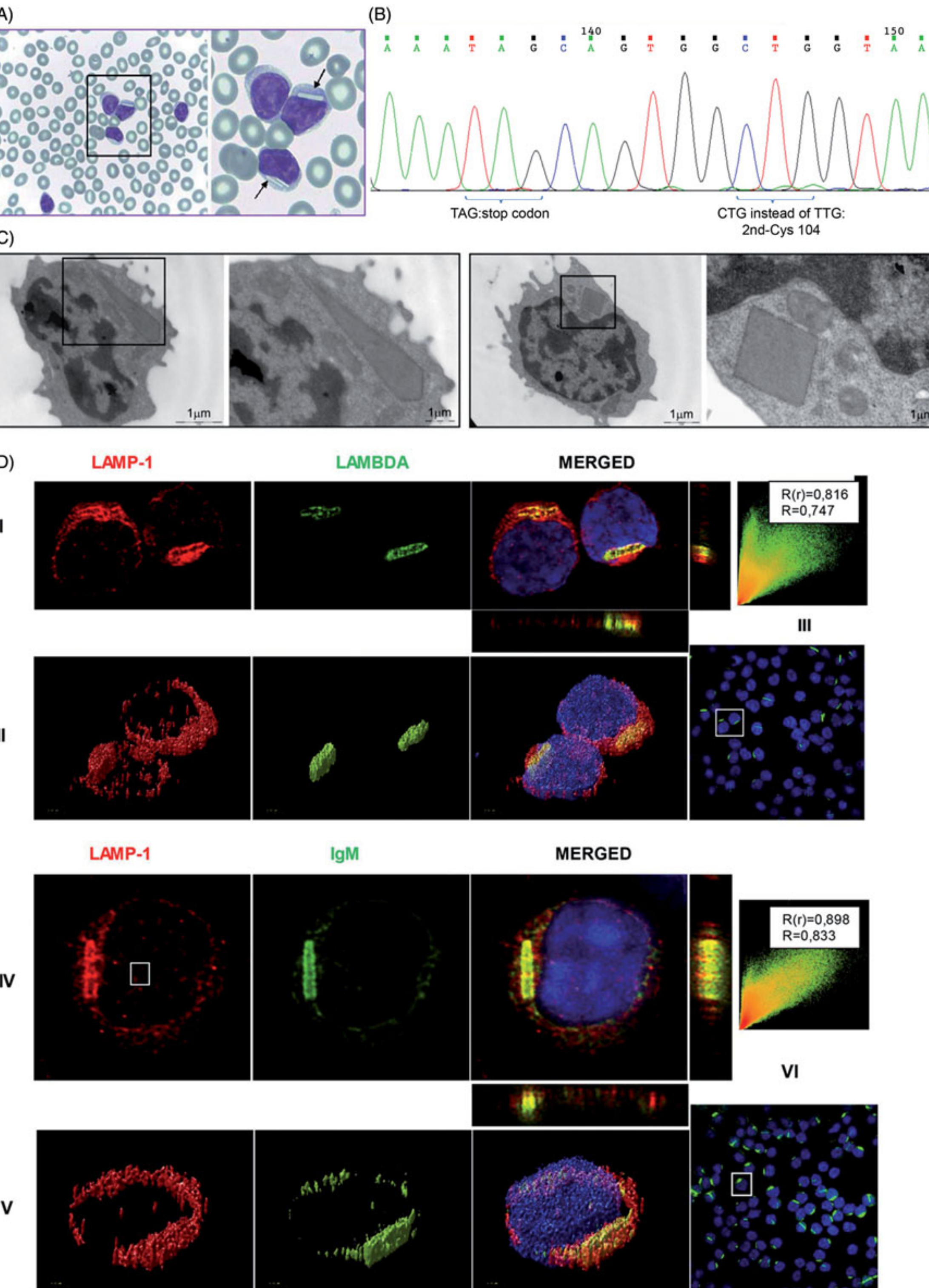

Figure 1. (A) May-Grünwald Giemsa staining of blood smear. Representative peripheral blood smears showing CLL lymphocytes containing cytoplasmic inclusions, ×1000. Black arrows show one or more inclusion per lymphocyte; (B) *IGHV* sequence showing the stop codon and the mutation T > C producing the absence of IMGT 2nd-Cys 104; (C) transmission electron microscopy of B CLL cells. Low and high magnification EM imagines of three different B cells showing crystal inclusions; (D) immunofluorescence evaluation of CLL lymphocytes containing cytoplasmic inclusions. Cytospin slides of CLL cells were immunostained with anti-lambda (I) or anti-IgM (IV) (green) and anti-LAMP-1 (red) antibodies. DAPI staining (blue) was used for nuclear visualization. Images shown are z-stack projections. (II) and (V) are 3D surface-rendered projections of the images above. Merged images and orthogonal views show co-localization between Lambda or IgM and LAMP-1 (yellow). (III) and (VI) show the area selected for posterior image acquisition. Scatter plots represent co-localization analyses between Lambda or IgM and LAMP-1 using SVI Huygens Essential 14.1 software. Pearson coefficient (*R*) and overlap coefficient (*R*[*r*]) are listed.

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CD38 positive leukemic cells. The cytogenetic analysis presented a normal karyotype: 46,XX, while fluorescence *in situ* hybridization (FISH) study showed trisomy 12 and 13q14 deletion (7.4% and 11.3% of cells, respectively). *IGHV* mutational status was also performed according to previously described [7]. PCR reaction was done on cDNA using primers that anneal to sequences in the leader region (LH1–LH6) and antisense Cμ-primer, and also using genomic DNA and standardized BIOMED-2 PCR protocol, as was recommended for problematic cases [8]. By both methodologies, we found two *IGHV* rearrangements: one unproductive comprising the segments *IGHV3-13*03*, *IGHD4-23*01*, and *IGHJ4*02*, carrying a stop codon and missing IMGT 2nd-Cys 104 (Figure 1(B)), and the other productive expressing *IGHV3-48*03*, *IGHD3-10*01*, and *IGHJ1*01*, both mutated (85.7% and 94.4% homology with the germ line, respectively). Due to its unfit condition, the patient was treated with chlorambucil and after two cycles, she achieved partial response. Currently, the patient continued in partial remission and presents no disease – related symptoms.

In addition, it was of our interest to analyze more specifically the characteristics of crystal inclusions observed in our patient. Thus, we have used transmission electron microscopy (TEM) to study their ultrastructural features and confocal microcopy to analyze the inclusions composition. TEM studies revealed one or more electron-dense, rectangular or rhomboid crystalline structures within lymphocytes as well as a high density of ribosomes in the cytoplasm. However, we could not definitely identify a clear RER or another type of limiting membrane around the crystals, as suggested in previous reports [3,6] (Figure 1(C)). Simultaneously, lymphocytes cytospins were stained with anti-IgM or anti lambda light chain (Dako, Glostrup, Denmark) and anti-lysosomal associated membrane protein 1 (LAMP-1, Abcam, Cambridge, UK) antibodies. The analysis by confocal microscopy showed that the crystals contained IgM and lambda light chain, displaying an identical pattern of heavy and light chains restriction to that found on the cell surface. These findings are in agreement with previous reports showing that intracytoplasmic inclusions in leukemic lymphocytes consist of monoclonal heavy and light chains Ig, and are usually coincident with their expression on the cell surface (Table 1). Furthermore, we have observed the presence of LAMP-1 around the crystals and also detected a high degree of co-localization between IgM or lambda light chain with LAMP-1, suggesting a lysosomal localization for these inclusions (Figure 1(D)) not previously reported in CLL. Different authors have shown similar TEM images than those observed in our patient, proposing locations in the RER, at the perinuclear space or bonded to the membrane (Table 1) but no studies with confocal microscopy to confirm these sites were performed, indicating the importance of this type of analysis to deepen the knowledge about these inclusion bodies.

Little is known about the precise mechanisms of formation of these structures. Some authors have suggested that their presence could be related to failures in the intracellular transport or to defects in the post-reticulum process of the IgMλ molecules [9,10]. Further studies [3,11] found unusual structural characteristics of the Ig and suggested that the inability of cells to secrete it might be responsible for the formation of these inclusions. In addition, biosynthetic studies have shown that intracytoplasmic Ig could precipitate as abnormal heavy chain due to a particular set of physicochemical properties, an imbalance between light and heavy chain or abnormal glycosylation [12–14]. More recently, Hasegawa [14] have proposed the existence of two different types of intracellular crystallization events, associated to distinct mechanisms of inclusion formation: one in the RER, related to protein synthesis, and the other in the endosome/lysosome compartments, associated to protein degradation, as was reported in plasma cell disorders [15]. Interestingly, as observed in Table 1, most of CLL cases with intracytoplasmic crystals showed IgMλ type (63%). Thus, our patient with a similar composition adds weight to the suggestion that the structure of this protein could lead to inclusions formation [10].

Additionally, there is scarce information about the molecular characteristics of the Ig that participate in crystals formation. Thus, we have studied the mutational status and the type of *IGHV* rearrangements present in our patient. This analysis showed two different *IGHV/IGHD/IGHJ* rearrangements one unproductive carrying a stop codon (which leads to the interruption of the protein transcription) and the absence of the 2nd-CYS 104, and another productive, that would support the presence of a biallelic condition. As known, the IGMT 2ndCys 104 is considered a VH CDR3 (*variable heavy complementarity determining region 3*) 'anchor', and their absence is critical for ensuring the integrity of this region. This type of rearrangement has rarely been found in repertoires from normal, autoreactive or malignant B cells, including CLL, and the functionality of rearrangements lacking this amino acid is unknown [8]. Interestingly, this is, to the best of our knowledge, the first CLL case exhibiting cytoplasmic Ig inclusions in which the *IGHV* mutational status was evaluated. The evaluation of *IGHV* rearrangements in more CLL patients with these structures may elucidate if any type of association exists.

Currently, the prognostic significance of these inclusions is unknown, but they do not appear to have clinical impact, as observed in our case. Nevertheless, studies analyzing the clinical outcome in an increased number of CLL patients with these precipitates could shed light on their role in this pathology.

Concluding, our findings provide new evidence about crystal bodies in CLL contributing to a better characterization of these particular structures. Overall, we have demonstrated the association between IgMλ intracytoplasmic precipitates and lysosomes and described the *IGHV*

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Table 1. Intracytoplasmic inclusions in CLL patients.

No. of patients	Type of inclusion	% of lymphocytes containing inclusions	Inclusion Ig class	Surface Ig class	Cellular location	Reference
1-1st study 2nd study	Crystal	60–70	IgAλ	IgM and IgG κ, λ	RER	[4]
1	Crystal	60–70	IgMλ	IgMλ		
1	Crystal	10–20	IgMλ	IgMλ		
1	Crystal	60–70	IgAλ	None		
1	Crystal	60	IgMλ	None	–	[12]
1-1st study 2nd study	Crystal	5–10	– IgGλ	IgGλ None	RER	[16]
14	Rod like crystals/ amorphous vesicular	1–41 54 40 44	11 IgMλ 1 IgAλ 1 IgGλ 1 IgMκ	9 like cytopl Ig/ 1 None/2 ND/ 2 IgM,D,λ	RER	[3]
1	Dense material	60	IgGκ	IgGκ	Membrane	[11]
1	Granular	41–47	IgMκ	IgM,D,κ	RER/Perinuclear	[13]
1	Round/oval	80	IgGκ	IgG,D,κ	Perinuclear	
1		70	λ	IgDλ	Perinuclear	
10	Granular	5–10 5 45 20	5 IgMλ/2ND ND ND ND	7 IgMλ 1 IgMκ 1 IgGλ ND	RER	[6]
1	Spherical eosinophilic	>50	IgMλ	IgMλ	RER	[17]
1	Filamentous	84–86	IgGκ	None	Perinuclear space, RER	[5]
1	Vermiform filamentous	31	IgGλ	–	RER	[2]
1	Filamentous	20	–	IgMλ	RER	[18]
4	Rod like	1–31	IgMλ	IgMλ	RER	[10]
1	Rod like	28	IgMλ	IgMλ	–	[9]
1	Filamentous like	50	IgMλ	IgMλ	–[a]	Present study

Ig: immunoglobulin; RER: rough endoplasmic reticulum; ND: non determined.
[a]Co-localization between IgM or lambda light chain with lysosomal associated membrane protein 1 (LAMP-1).

rearrangements observed in a CLL patient with crystal inclusions. The significance of this particular location remains uncertain and more studies will be necessary to clarify this point.

Acknowledgements

This work was supported by grants from the National Agency of Scientific and Technical Promotion (ANPCyT) (PICT N°: 2014-1566), CONICET (National Council of Scientific and Technical Researches) (PID N° 1122015 0100753) and from the National Cancer Institute of Argentina.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article online at https://doi.org/10.1080/10428194.2017.1370549.

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