Autoimmune conditions and chronic infections in chronic lymphocytic leukemia patients at diagnosis are associated with unmutated IgV$_H$ genes

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ABSTRACT

Few data are available concerning the prevalence of autoimmune disease or chronic infections in chronic lymphocytic leukemia patients at diagnosis as well as their clinical outcome. We studied the frequency of such chronic conditions in relation to prognostic markers. A history of autoimmune disease or chronic infection was found in 21% of 186 chronic lymphocytic leukemia patients (12% in autoimmune diseases, 9% in chronic infections). Patients with a history of chronic stimulation were more likely to have unmutated IgV$_H$ genes (p<0.002), unfavorable or intermediate risk cytogenetics (11q, 17p deletions, trisomy 12) (p<0.001), and higher CD38 expression (p=0.004). Autoimmune conditions (n=22) were characterized by female predominance (55.0%) with a high frequency of unmutated IgV$_H$ (53,8%). Median time to first treatment was 83 months for the chronic stimulation group compared to 128 months for the non-chronic stimulation group (n.s.). Patients suffering from chronic conditions at chronic lymphocytic leukemia diagnosis are likely to have poor prognostic markers, particularly unmutated IgV$_H$ genes.

Key words: chronic lymphocytic leukemia, chronic antigenic stimulation, IgV$_H$ mutation status, cytogenetics.

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Introduction

B-cell chronic lymphocytic leukemia (CLL) is a clonal expansion of B cells. Little is known about underlying factors or specific triggers,[1] although it seems clear that survival of CLL cells is associated with stimulation of and signaling through the B-cell receptor (BCR).[2] Leukemic cells are characterized by strikingly similar rearrangements of the immunoglobulin (Ig) variable region genes (IgV$_H$) which are biased compared to normal B cells.[3,4] The skewed V$_H$ gene usage, possibly caused by selective pressure via the BCR, is thought to give survival advantage by constant antigenic stimulation of the leukemic clone. Thus, antigens are thought to play a role in the pathogenesis of CLL.[2,5] Recent evidence based on Ig usage and sequence suggests that at least some CLL clones are derived from autoreactive precursors.[6] This has been corroborated by functional experiments.[7-10] Despite increasing evidence for the involvement of chronic antigenic stimuli based on IgV$_H$ sequence analysis, data on obvious clinical association with autoimmune diseases (AI) or chronic infections (CI) at diagnosis are rare. One study reporting autoimmune complications (autoimmune hemolytic anemia, thrombocytopenia, or similar) in CLL patients found 16% of patients, mostly in Binet stage A, to suffer from clinically overt autoimmune diseases.[11] The relationship between chronic clinical conditions and molecular or cytogenetic markers in CLL has never been studied. The data reported here provide novel evidence for the association of AI or CI in CLL patients at diagnosis with mutational status and cytogenetic aberrations.

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The online version of this article contains a supplementary appendix.

Conclusions regarding direct involvement of chronic stimulation in the development of CLL cannot be drawn from our study. However, our data suggest that patients with pre-existing autoimmune conditions or chronic infections are more likely to have high-risk disease.

Design and Methods

Patients

The charts of CLL patients seen between 1991 and 2006 at the Division of Haematology and Haemostaseology at the Vienna General Hospital were reviewed retrospectively for information on chronic stimulation at or before diagnosis of CLL. Selection was based on availability of well kept chart reviews and known IgV$_H$ mutation status. The majority of patients (75%) were first seen between 2001 and 2006 (median observation period: 53 months). Written informed consent was obtained for collection of blood cells for the determination of molecular parameters (Ethics approval numbers 38/1998, 505/2002, 495/2003). A diagnosis of autoimmune disease was made based on clinical manifestations. Chronic infections were defined as chronic infection or recurring history of an infection of more than six months. For patient selection refer to the algorithm in Figure 1 and the detailed description in the *Online Supplementary Appendix Methods*. The final number of patients included in the analysis was 121, 39 patients with chronic stimulation (CS group) and 82 patients without chronic stimulation (non-CS group).

Determination of IgV$_H$ gene usage and mutation status

Genomic DNA from MNC and BM samples was isolated using the MagNA Pure LC DNA isolation system (Roche). PCR amplification of clonal VDJ rearrangements was performed as previously described.[12] Sequences were analyzed using IMGT/V-QUEST (*http://imgt.cines.fr/IMGT_vquest/vquest?livret=0&Option=humanIg*) to identify most closely related germline fragments and homology to the nearest germline fragments was calculated.[12,13] More detailed information and primer sequences are given in the *Online Supplementary Appendix Methods.*

Fluorescence in situ hybridization

Genetic aberrations were assessed by fluorescence *in situ* hybridization (FISH) as described.[14]

Statistical analysis

χ^2 test and Fisher's exact t-test were used to compare categorical data. Student's t-test was used to compare patient groups with respect to continuous, normally distributed data. Time to first treatment and overall survival were analyzed using Kaplan-Meier estimation and log-rank functions in SPSS (15.0).

Results

Entire patient cohort

General considerations and subgroup definitions

Patient and data selection is shown in the flow chart of Figure 1. Within the data set with available information on IgV$_H$ gene usage and mutation status (n=186), the incidence of chronic stimulation was 20.9% (n=39), 22 patients suffering from AI (11.8%) and 17 from CI (9.1%). AI included autoimmune thyroiditis/Hashimoto's thyroiditis (n=5), psoriasis (n=5), vasculitis (n=4), polyneuropathy (PNP, CIDP) (n=3), allergic asthma (n=2), celiac disease (n=1), rheumatoid arthritis (n=1), and sarcoidosis (1). CI included *Helicobacter pylori* – gastritis (n=5), chronic sinusitis or laryngitis (n=4), chronic respiratory tract infections (n=3), recurring herpes infections (*Herpes/Varicella-Zoster/Cytomegalovirus*) (n=2), chronic colitis (n=1), hepatitis C (n=1), and recurring pneumonia (n=1). There was no difference in the distribution of CS patients and IgV$_H$ mutation status before and after 2001 (*data not shown*). Six of the 22 AI patients had received immunosuppressive therapy before CLL was diagnosed; one of these has received treatment for CLL.

In order to remove any bias, we excluded all patients

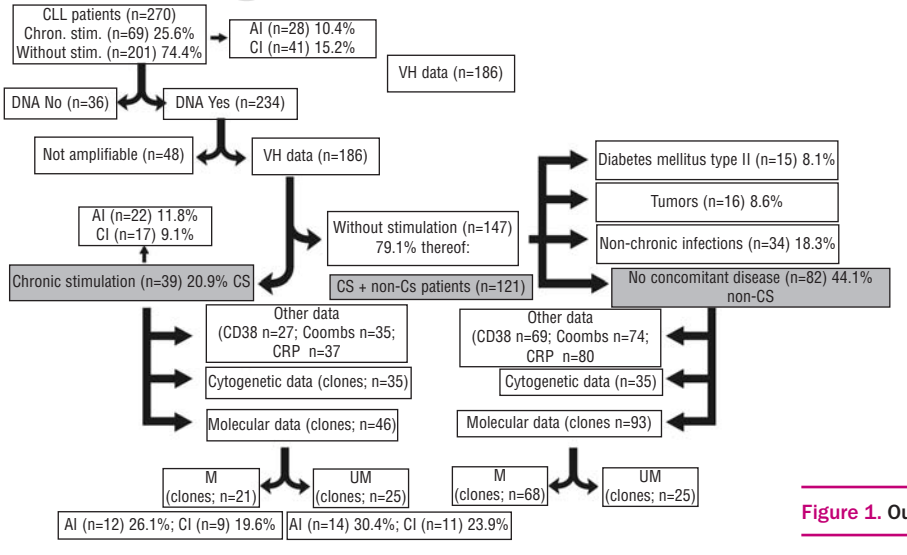

Figure 1. Outline of the patient selection process.

Table 1. Characteristics of chronic stimulation and non-chronic stimulation chronic lymphocytic leukemia patients.

	Chronic stimulation		*Non-chronic stimulation*		*p**
Median age/range	60	25-79	62	39-83	n.d.
Gender: male (%)/female (%)	56.4	43.6	64.6	35.4	n.s.
AI: male (%)/female (%)	45.0	55.0			
CI: male (%)/female (%)	68.4	31.6		female AI *vs.* CI	n.s.
Binet stage (CS N=39 ; non-CS n=82)					
A (n/%)	32	82.1	73	89.0	n.d.
B (n/%)	5	12.8	7	8.5	
C (n/%)	2	5.1	2	22.4	
CD38 expression (CS N=27; non-CS n=69)					
Median/range	23	1-79	4	0-85	0.004
Coombs test (CS N=35; non-CS n=74)					
Positive (n/%)	2	5.7	4	5.5	n.d.
Negative (n/%)	33	94.3	70	94.6	
CRP (CS n=37; non-CS n=80)					
<0.5 (n/%)	29	78.4	62	77.5	
≥0.5 (n/%)	8	21.6	18	22.5	
Median/range	0.57	0.16-14.14	0.53	0.04-2.18	n.d.
Mutation status (clones: CS n=46; non-CS n=94)					
UM (%)/M (%)	54.3	45.7	27.7	72.3	0.002
Mutation status (clones: CS n=46; non-CS+DM+tumors+incurrent infections n=174)					
UM (%)/M (%)	54.3	45.7	32.8	67.2	0.007

Genetic abnormalities (CS n=35; non-CS n=65)			AI (N)	Inf (N)			
Normal (n/%)	6	17.1	4	2	17	26.2	n.s.
13q- (n/%)	14	40.0	5	9	38	58.5	0.036
143q- sole (n/%)	4	11.4	2	2	21	32.3	0.021
11q- (n/%)	11	31.4	5	6	7	10.8	0.009
+12 (n/%)	10	28.6	5	5	6	9.2	0.01
17p- (n /%)	3	8.6	1	2	5	7.7	n.s.
14q32 rearr. (n/%)	5	14.3	2	4	15	23.1	n.d.
Other (6q- etc) (n/%)	4	11.4	2	2	1	1.5	n.d.

*CS: chronic stimulation; *n.s.: not significant; n.d. : not determined.*

from the non-CS group suffering from concomitant disease (diabetes mellitus type II (DM), non-chronic infections, or tumors at some point in their lives), while in the CS group we considered long-term chronic antigenic stimulation to outweigh concomitant or intermittent disease and did not exclude such patients. This resulted in a final 121 patient cohort, with n=39 (clones: n=46) in the CS group and n=82 (clones: n=94) in the non-CS group (Figure 1). As shown in the *Online Supplementary Results Appendix,* Table 1, the baseline characteristics of this final patient cohort represent an average CLL population.

IgV$_H$ gene usage

V$_H$1-69 was the most frequently used gene (11.4%), followed by 4-34 (8.6%), 3-30 (7.9%), 3-23 (6.4%), 3-7 (5.7%), 5-51 (5.0 %), and 2-5 (5.0%). This distribution is typical for V$_H$ gene usage in CLL.[2] Detailed V$_H$ gene usage is shown in supplementary results in the *Online Supplementary Results Appendix ,* Figure 1A.

Characteristics of chronic stimulation- and non-chronic stimulation groups

Molecular genetics and gender differences

Patients with a history of CS had significantly more UM V$_H$ genes (54.3% *vs.* 27.7%; p<0.002), less 13q and 13q sole abnormalities (p=0.036 and 0.021, respectively), more 11q deletions (p=0.009), more trisomy 12 (p=0.01), and higher expression of CD38 (median 23% *vs.* 4%; p=0.004) (Table 1). They also had significantly more often at least one of the aberrations associated with more rapid disease progression (11q deletion, 17p deletion, trisomy 12) when patients with concomitant 13q deletions were excluded (p<0.001). Women in the CS group had a higher percentage of UM clones when compared to female non-CS patients (23.4% *vs.* 4,3%; p=0.002). In the non-CS group, men were more likely to have UM IgV$_H$ genes compared to women (23.7% *vs.* 4.3%; p=0.01) (*Online Supplementary Results Appendix,* Table 2).

V$_H$ gene usage and mutation status

The most frequently used V$_H$ genes were 4-34, 1-69, 3-30, and 5-51 for the CS group and 1-69, 3-23, 3-7, 3-30, and 4-34 for the non-CS group. No statistical difference between CS and non-CS patients was observed regarding V$_H$ gene usage (*Online Supplementary Results Appendix, Table 3 and Figures 1B and 1C*). We also compared IgV$_H$ gene rearrangements of previously described stereotyped receptors with IgV$_H$ sequences found in our

CLL cohort.[3,4,15] Positive matches were found in 18.6% of CS patients and 23.7% of non-CS patients (*Online Supplementary Results Appendix, Table S4*). While only Ig heavy chains and no light chains were analyzed, there was no obvious difference between CS and non-CS patients regarding subclasses.

Clinical outcome

Patients with CS showed a tendency towards earlier treatment (Figure 2). However, median Kaplan Meier estimate of time to first treatment (TFT) between CS and non-CS patients was not significantly different (TFT CS 83 months *vs.* non-CS 128 months; p=0.3). Overall survival was not calculated because to date only 2 patients have died.

Comparison of autoimmune disease and chronic infection in the chronic stimulation group

Mutational status was almost equal between the 22 patients (26 clones) with AI and the 17 patients (20 clones) with CI (UM 53.8 *vs.* 55.0% clones). While there was a higher number of female patients in the AI group compared to the CI group (12/22=54.5% *vs.* 5/17= 29.4%), this was not statistically different. Female AI patients had a high proportion of unmutated IgV$_H$ genes (53.8%).

Patients excluded from final analysis in the non-chronic stimulation group

Patients not included in the non-CS group were those who had concurrent DM, cancer, or non-chronic infections. No particular features in V$_H$ gene usage could be found in these patients (*data not shown*). Considering mutation status only (*Online Supplementary Results Appendix, Table S5*), patients with metabolic disease or tumors had 31.8% and 26.3% unmutated V$_H$ genes respectively. Of note, patients with non-chronic infections and other illnesses had 47.5% unmutated V$_H$ genes. Including patients with DM, tumors, and incurrent infections into the non-CS group, the percentage of UM IgV$_H$ genes was 32.8% *vs.* 54.3% for the CS group (p=0.007).

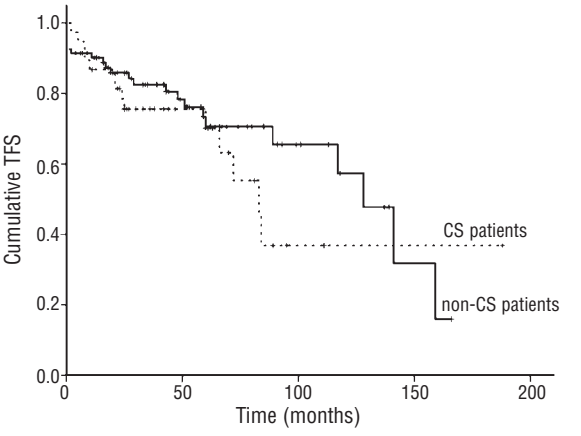

Figure 2. Kaplan-Meier plot comparing treatment-free survival of CS and non-CS patients.

Discussion

We show that autoimmune diseases and chronic infections are present at the time of CLL diagnosis at a frequency of 21% (12 and 9% respectively). While our findings do not allow the conclusion that chronic stimulation directly induces development of CLL, the data suggest that the presence of a chronic condition at diagnosis is associated with poor prognostic markers.

Autoimmune complications (AIHA, ITP) in the course of the disease have been reported for many years (*see Hamblin[16] for summary*). An association of chronic stimulation at or before the onset of CLL, however, has rarely been documented. Data on the association of AI with an increased risk of developing CLL are scarce.[17-19] Clinically, Barcellini[11] found 16% of CLL patients to suffer from AI. Other than disease stage, no clinical or prognostic data were included in this study. UM V$_H$ status was related to the occurrence of ITP and poor survival.[20] While in our study 11.8% of CLL patients suffered from various autoimmune diseases, our data do not suggest that any of the diseases bear an increased risk for CLL *per se*. Clinically overt autoimmune diseases are estimated to occur with a frequency of 3-5% in Western countries.[21] This implies that, in our cohort, autoimmune conditions are 2 to 3-fold over-represented at diagnosis. No difference was found concerning the impact of hepatitis C infection on CLL presentation and outcome.[22] However, an association was found of pre-existing infectious disease, particularly chronic respiratory tract infections and herpes virus infections, with CLL.[19] These two entities were also among the most frequent CIs in our cohort (9/17).

Our data suggest that patients with pre-existing autoimmune conditions or chronic infections are more likely to have high-risk disease. CS patients were characterized by the association of known poor risk factors, particularly UM V$_H$ genes.[12,23] Median time to first treatment was shorter in CS patients compared to non-CS patients, albeit not statistically significant. This may be due to the short observation time and low patient numbers. Of note, some AI patients remained untreated for many years.

Twenty percent of BCRs in B-CLL are stereotyped.[4] In our study, the heavy chain fraction of stereotyped receptors was not more frequent in the CS group suggesting no major overlap between the subgroup of patients with clinically overt CS and patients with stereotyped receptors. Our approach has some limitations and draw-backs. Patient subgroups were defined by chart review relying on the accuracy of clinical records. Reports on AI and CI were carefully reviewed under strict criteria and corroborated by medical records. Cut-offs for duration of CI may vary with the underlying disease. For pragmatic reasons we chose six months for this study. Other researchers have used one year.[19] The number of CS patients may be underestimated due to missing information. Direct serological evidence for AI was not obtained in all patients. Other researchers have approached the problem by

reviewing patient diagnosis through ICD codes.[19] In both cases, confidence is placed in the diagnosing doctor and medical records on AI provided by the patient during the interview. While we have described patients at the earliest available diagnostic work-up, in some cases it remains unclear whether CLL was not present earlier leaving the possibility that infections were a result of immunosuppression by CLL and not pre-existent.

CLL shows sex-specific distribution of risk factors. Men are much more likely to have UM V_H genes and a shorter survival.[24] In our study, female CS patients show more frequently UM IgV_H genes compared to female non-CS patients. This difference is highly significant (p=0.002) due to a higher percentage of women among AI patients (54.5%). This is in contrast to all other CLL subgroups, but consistent with sex distribution in AI. The distribution of UM and M IgV_H genes is statistically different between men and women in the non-CS group (p=0.01) with women having preferentially mutated genes.

In summary, chronic antigenic stimulation seems to occur at considerable frequency in CLL patients at or before the onset of leukemia. We document the association of CS with UM V_H status and other poor risk factors. Further studies regarding clinical outcome and impact of CS on the biology of disease are warranted.

Authorship and Disclosures

KV designed the research, collected and analyzed patient data, wrote the paper; TL, FS, EP collected and analyzed patient data; KE, AH, CSk, AG, ChSt diagnosed and treated the patients and provided clinical data; HE, EK, IS, BS, CF, MS, OW, SSt performed diagnostic and laboratory work; UJ initiated, designed and supervised research, analyzed data, and wrote the paper. All authors reviewed the manuscript critically for important intellectual content and approved the final version. The authors reported no potential conflicts of interest.

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