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Malignant Lymphomas

Interleukin-1 β, interleukin-1 receptor antagonist and interleukin-6 plasma levels and cytokine gene polymorphisms in chronic lymphocytic leukemia: correlation with prognostic parameters

JANNE HULKKONEN,* JUHANI VILPO,° LEENA VILPO,° TOMI KOSKI,° MIKKO HURME*
**Department of Microbiology and Immunology, University of Tampere Medical School, Tampere, Finland; °Laboratory of Molecular Haematology, Department of Clinical Chemistry, Tampere University Hospital and University of Tampere Medical School, Tampere, Finland*

ABSTRACT

Background and objectives. **The growth of B-cell chronic lymphocytic leukemia (B-CLL) cells has been shown to be dependent on exogenous growth factors *in vitro*. We wanted to evaluate the clinical relevance of interleukin (IL)-6, IL-1β and interleukin-1 receptor antagonist (IL-1Ra) in B-CLL. As the plasma levels of IL-6, IL-1β and IL-1Ra have been suggested to be partly dependent on gene polymorphism, the previously described polymorphisms of the IL-1 complex genes and the IL-6 gene were also studied.**

Design and methods. **The plasma levels of these cytokines were measured in a cohort of 36 patients with B-CLL and in 400 healthy subjects. The previously described polymorphisms of the IL-1 complex genes and the IL-6 gene were studied using PCR and RFLP. These data was correlated with other parameters associated with severity and prognosis of B-CLL and a number of clinical and laboratory findings.**

Results. **The plasma concentrations of IL-1β and IL-1Ra were lower in B-CLL patients than in normal controls ($p < 0.001$). The IL-1β plasma levels were dependent on the cell immunophenotype score and state of progression of the disease. Moreover, plasma concentrations of IL-6 were elevated in B-CLL patients compared with healthy subjects ($p < 0.005$) and correlated with disease stage, hemoglobin levels, anemia and erythrocyte sedimentation rate in the patients. The allele frequencies of the analyzed genes were similar in patients and controls.**

Interpretation and Conclusions. **Our data demonstrate that in B-CLL, plasma levels of IL-1β, IL-1Ra and IL-6 differ from normal, and mechanisms other than allelic imbalance of their genes account for the distinct cytokine profiles observed in this disease.**

Key words: interleukin-1, interleukin-1Ra, interleukin-6, polymorphism, CLL

Correspondence: Janne Hulkkonen, M.D., University of Tampere Medical School, Department of Microbiology and Immunology, P.O. Box 607, FIN-33101 Tampere, Finland. Phone: international +358-3-2157141 – Fax: international +358-3-2156173 – Email: bljahu@uta.fi

Chronic lymphocytic leukemia of the B-cell type (B-CLL) is a disease characterized by expansion of mature-appearing B-lymphocytes in the peripheral blood and bone marrow. Recently published data support the theory that the events involved in the expansion of a malignant B-cell clone may be dependent on exogenous soluble growth factors. Cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-2, IL-4, IL-6, IL-8, IL-10 and interferon (IFN)-α have been proposed to play a role in the activation, growth and apoptosis of leukemic B-cells.[1-3] Plasma levels of IL-6 have been shown to be elevated in B-CLL in a stage-dependent manner.[4] IL-1 is a potent inducer of IL-6 and it has been suggested that the circulating level of IL-6 is the best marker of elevated IL-1 activity in most disease states.[5] On the other hand, interleukin-1 receptor antagonist (IL-1Ra) production is partly dependent on endogenous IL-6 production.[6]

The inducible responses and the basal production of IL-1α, IL-1Ra and IL-6 proteins have been suggested to be dependent on the allelic state of cytokine genes. It has been found that a Taq I polymorphism in the human interleukin-1β gene correlates with IL-1β secretion *in vitro*.[7] Others and ourselves have recently demonstrated that the individual variability in IL-1Ra plasma levels can be explained by IL-1β and IL-1Ra gene polymorphisms.[8,9] Recent findings suggest that the transcriptional activity of the interleukin-6 gene and the plasma levels of IL-6 protein are associated with a single base exchange polymorphism sited at the 5' flanking region of the IL-6 gene.[10] Imbalances in these polymorphisms are seen in several inflammatory and autoimmune diseases.[10,11] Several reports have also emphasised that these polymorphisms can affect the outcome and mortality rates of hematologic malignancies.[12]

We previously observed that the production of IL-6 *in vitro* varies significantly among patients with different stages of chronic lymphocytic leukemia.[13] In this study we have analysed IL-1β, IL-1Ra and IL-6 plasma levels and the polymorphisms of these genes in B-CLL patients and normal controls. The analysed polymorphisms and the measured cytokine levels were compared with several clinical and laboratory parameters in order to find out whether these variables are associated with the severity of disease, or predispose patients to B-CLL-associated complications.

Design and Methods

Patients

Clinical specimens were obtained after informed consent from 36 consecutive patients referred to the CLL outpatient clinic of Tampere University Hospital. Clinical and hematologic data are presented in Table 1. The diagnosis and staging of B-CLL were based on standard clinical, morphologic and immunophenotypic criteria as described in detail elsewhere.[13] The B-cell nature and the cell immunophenotypic score of all B-CLL cases were confirmed as described previously.[14] Cases #9 and 28 had previously been treated with Leukeran (chlorambucil) and cases #8, 10, 24 and 32 with Leukeran and prednisolone. Cases #2, 12 and 21 had also been treated with more intensive protocols comprising cyclophosphamide, adriamycin, vincristine and prednisolone. The state of progression of the disease was evaluated on the basis of annual lymphocyte doubling time (*slow*: annual increase in lymphocyte count less than 20%; *intermediate*: annual increase of 20-100%; *fast*: annual increase over 100%).

Normal controls

Blood samples from normal healthy controls were obtained from the Finnish Red Cross Blood Transfusion Centre, Tampere.

Blood samples and DNA isolation

Blood samples were obtained and peripheral blood mononuclear cells were separated as described previously.[15] DNA was isolated from mononuclear cells by using the salting out method.[16]

Genotype analysis

Base exchange at position -889 of the IL-1α gene was analysed as previously described.[17] Oligonucleotides 5' AAGCTTGTTCTACCACCTGAACTAGGC 3' and 5'TTACATATGAGCCTTCCATG 3' flanking the polymorphic site were used as primers in polymerase chain reaction (PCR). For IL-1β analysis two different polymorphic regions were analyzed. The region that contains the Ava I polymorphic locus at position -511 of the IL-1β gene was amplified by PCR using oligonucleotides 5' TGGCATTGATCTGGTTCATC 3' and 5' GTTTAGGAATCTTCCCACTT 3' as primers.[18] The Taq I polymorphic region at exon 5, position +3953 of the IL-1β gene was amplified by PCR using oligonucleotides 5' GTTGTCATCAGACTTTGACC 3' and 5' TTCAGTTCATATGGACCAGA 3' as primers.[7] The Nla III polymorphic site at promoter region position -174 of the IL-6 gene was amplified by PCR using oligonucleotides 5' TGACTTCAGCTTTACTCTTGT 3' and 5' CTGATTGGAAACCTTATTAAG 3'.[10] After restriction enzyme digestion the products of the PCR analyses were identified by electrophoresis (on 9% PAGE) and ethidium bromide staining. The IL-1Ra exon 2 polymorphism caused by 86-bp tandem repeats was analyzed as described previously,[19] using oligonucleotides 5' CTCAGCAACACTCCTAT 3' and 5' TCCTGGTCTGCAGGTAA 3' as primers and 2% agarose gel for detection of amplified DNA products. Cycling conditions for the primer pairs were analogous to those published previously (references above).

ELISAs

Plasma interleukin-1β, IL-1Ra and IL-6 concentrations were determined by using commercially available enzyme-linked immunosorbent assays (Pelikine Compact human IL-1β and human IL-6 ELISA kits, CLB, Amsterdam and Quantikine human IL-1Ra immunoassay, R&D systems, Minneapolis, USA), following the manufacturer's instructions. The optical density of individual wells was determined with a Multiscan Biochromatic 348 (Titertek) spectrophotometer. The detection limits of the assays were 0.4 pg/mL for IL-1β, 0.6 pg/mL for IL-6 and 46.9 pg/mL for IL-1Ra.

Table 1. Patients: clinical and hematologic data at the time of sampling.

No.	Age/sex (yrs)	FAB-diagnosis*	Binet stage	PRG†	Immuno phenotype°	Blood lymph. (10^9/L)
1	61/F	CLL	A	S	NA	86
2	58/M	CLL/PL	C	T	4 (SmIg)	310
3	68/M	CLL	B	S	3 (CD23,CD5)	66
4	68/M	CLL	A	I	5	54
5	50/F	CLL/MIX	A	S	4 (SmIg)	130
6	72/M	CLL	B	F	4 (SmIg)	84
7	55/M	CLL	A	S	5	64
8	65/M	CLL	A	T	5	59
9	68/F	CLL	C	T	5	88
10	65/M	CLL	C	T	5	132
11	68/M	CLL	C	F	3 (SmIg,CD5)	114
12	67/F	CLL/PL	C	T	4 (SmIg)	188
13	68/F	CLL	A	I	5	81
14	57/M	CLL	A	F	5	120
15	73/F	CLL/MIX	C	F	3 (CD23,SmIg)	79
16	58/M	CLL/PL	A	F	4 (CD5)	36
17	53/M	CLL/PL	B	I	5	69
18	71/M	CLL/MIX	B	S	4 (SmIg)	89
19	55/M	CLL/PL	B	I	4 (SmIg)	59
20	65/M	CLL	B	S	3 (CD23, SmIg)	59
21	79/F	CLL/MIX	B	T	4 (CD23)	97
22	48/M	CLL	A	S	5	93
23	67/M	CLL	A	I	5	67
24	69/M	CLL	B	F	5	92
25	57/M	CLL/MIX	B	T	4 (SmIg)	198
26	60/M	CLL	A	F	5	63
27	58/M	CLL	A	T	4 (SmIg)	68
28	60/M	CLL	A	I	5	99
29	79/F	CLL	A	S	4 (SmIg)	155
30	77/F	CLL	C	F	5	206
31	76/M	CLL	B	I	4 (SmIg)	173
32	68/M	CLL	C	F	NA	178
33	72/M	CLL	A	F	3 (CD22,SmIg)	134
34	79/M	CLL	B	F	4 (CD5)	224
35	71/F	CLL	A	F	4 (SmIg)	69
36	63/M	CLL/PL	C	F	3 (SmIg,FMC7)	185

**FAB diagnosis: according to Bennett et al.[34]; †Pattern of progression. S (slow or no progression), I (intermediate) and F (Fast), T: chemotherapy given previously or natural disease progression non-evaluable. ‡Immunophenotype score: The system of Matutes et al.[14] was used. A Score of 5 means: CD22-, CD23+, CD5+, FMC7- and undetectable/weak SmIg κ or λ. Markers causing a deviation from score 5 are indicated in parentheses: for example (CD23) means CD23 negativity. Lymph.: lymphocytes; NA: not analyzed.*

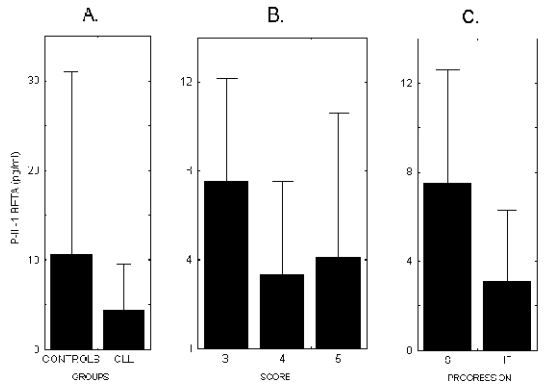

Figure 1. Panel A: Plasma IL-1β levels (means and SDs) in B-CLL patients (n = 34) and in normal controls (n = 400). Panel B: IL-1β plasma levels in B-CLL patients with different immunophenotype scores (score 3, n = 6; score 4, n = 13; score 5, n = 13). Panel C: IL-1β plasma levels in B-CLL patients having different forms of progressive disease (Slow/non-progressive, n = 7; intermediate-fast, n = 20). Cytokine concentrations are in picograms per milliliter.

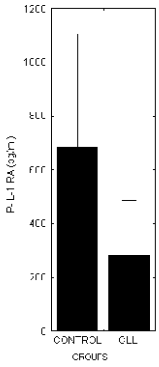

Figure 2. Plasma levels (means and SDs) of IL-1Ra in healthy controls (n = 200) and in B-CLL patients (n = 34).

Statistical analysis

Mean values were compared by using the Mann-Whitney U-test. Allele frequencies were compared by the χ^2 test and Fisher's exact test. Correlations were calculated using the Spearman rank order correlation test. Findings were considered statistically significant at $p < 0.05$.

Results

Interleukin-1β plasma levels

In 3 of the B-CLL patients (n = 34) and in 9 of controls (n = 400) the interleukin-1β plasma levels were below the assay limit. The IL-1β plasma levels were lower in B-CLL patients (4.37±5.25 pg/mL; mean± SD, median 2.71) than in the healthy controls (10.6 ±20.5 pg/mL; median 5.78, p<0.001) (Figure 1A). The IL-1β levels varied with the immunophenotype score (Figure 1B). They were 7.54±4.62 pg/mL (median 5.74) for score 3 patients; 3.34±4.20 pg/mL (median 1.32) for score 4 patients and 4.11±6.53 pg/mL (median 1.65) for score 5 patients (score 3 patients vs. score 4 or 5 patients $p < 0.05$). No single immunophenotypic marker was found to be associated with this result. The IL-1β levels were closer to normal in those patients who had non-progressive disease (7.49±5.12 pg/mL, median 8.25) compared with the progressive (intermediate and fast) group (3.09±3.17 pg/mL, median 2.67, $p < 0.05$) (Figure 1C). There were no statistically significant differences in IL-1β production among B-CLL patients when the Binet stage of the disease, FAB-classification or treatments previously given were used as categorizing parameters.

Interleukin-1Ra plasma levels

In 4 of the B-CLL patients (n = 34), interleukin-1Ra plasma levels were below the assay limit. The IL-1Ra plasma concentrations of healthy controls (n = 200) were all measurable with the applied methodology. As was the case with IL-1β, the observed IL-1 Ra plasma levels were clearly lower in B-CLL patients (281± 204 pg/mL, median 261) than in the normal controls (682±423 pg/mL, median 587, $p < 0.001$)(Figure 2). In contrast to the IL-1β results, IL-1Ra levels were lower in the non-progression group (207±85.73 pg/mL, median 202) compared with the fast plus intermediate progression group (325±245 pg/mL, median 313), although the difference was not statistically significant. No significant differences in IL-1Ra plasma levels were found within the B-CLL group when cell immunophenotypic score (Table 1), Binet stage, FAB-classification or treatments previously given were used to categorise the patients.

Interleukin-6 plasma levels

In 5 (n=36) of the B-CLL patients and 57 (n = 400) of the controls IL-6 plasma levels were below the assay limit. The plasma levels of IL-6 were elevated in B-CLL patients (3.75±7.53 pg/mL, median 1.82) compared within the normal controls (1.80±2.53 pg/mL, median 1.22, $p < 0.005$) (Figure 3A). The IL-6 levels were higher in Binet C patients (8.47±14.14 pg/mL, median 4.63) than in Binet B (3.13±3.05 pg/mL, median 2.56) or Binet A patients (1.51±0.79 pg/mL, median 2.36, A vs. C $p < 0.05$). The IL-6 plasma levels correlated negatively (R = –0.53, $p < 0.001$) with hemoglobin levels of B-CLL patients. Plasma IL-6 was higher in B-CLL patients with anemia (5.22±9.37 pg/mL, median 2.36) than in B-CLL patients without anemia (1.43±1.25 pg/mL, median 1.47, $p < 0.05$) (Figure 3B). This trend was seen independently at all Binet stages of the disease. A hemoglobin concentration of < 130 g/L for male and < 120 g/L for female subjects was used as the criterion for anemia. Plasma IL-6 levels correlated positively with the erythrocyte sedimentation rate (ESR; R = 0.41, $p < 0.05$) in B-CLL patients. As was the case with IL-1Ra, no correlation between cell immunophenotypic score (Table 1) or treatment, and IL-6 production was found.

IL-1Ra/IL-1β ratio

The mean IL-1Ra/IL-1β concentration ratio was 242±380 (median 114) for normal controls and 187±354 (median 70.6) for B-CLL patients. This ratio was lower (32.8±20.8, median 29.4) in the patient group having non-progressive disease com-

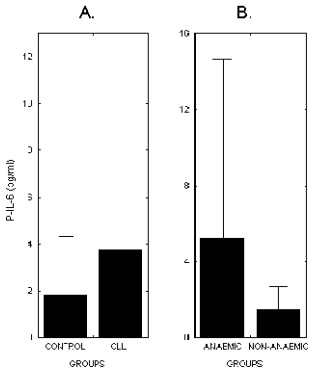

Figure 3. Panel A: Plasma levels (means and SDs) of IL-6 in healthy controls (n = 400) and in B-CLL patients (n = 36). Panel B: Plasma levels in anemic (n = 22) and non-anemic (n = 14) B-CLL patients.

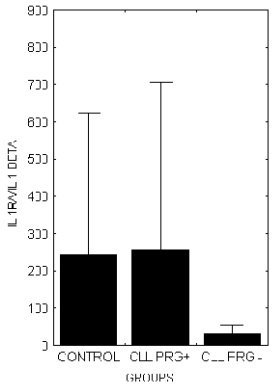

Figure 4. Ratios of IL-1Ra and IL-1β in plasma of normal controls (n = 400) and B-CLL patients (n = 34) with progressive (CLL PRG+)or non-progressive (CLL PRG-) disease.

pared within patients with fast or intermediate progressive disease forms (256±449, median 95.5). The difference in IL-1Ra/IL-1β ratios was significant ($p < 0.01$) between normal controls and non-progression B-CLL patients (Figure 4).

Allele frequencies

The allele frequencies among B-CLL patients and control subjects are summarized in Table 2. The distributions of the IL-1α, IL-1β (-511), IL-1β (+3953) and IL-1Ra alleles were very similar in the patient samples and the controls. The same was true when the frequencies of allele combinations formed on the basis of IL-1α, IL-1β and IL-1Ra genes were compared between patients and controls. No differences in allele frequencies were seen when the progression grade or Binet stage of the disease was applied as a grouping parameter (data not shown). As expected (see the introduction), the IL-1β and IL-1Ra plasma levels varied depending on the allelic state of the subjects (Figure 5). In normal controls, IL-1β plasma levels were significantly higher among IL-1β (-511) allele 2 carriers than among non-carriers ($p < 0.05$). The same trend was seen in plasma levels of IL-1β in IL-1β (+3953) allele 2 carriers and non-carriers and in IL-1Ra plasma levels between IL-1Ra allele 2 carriers and non-carriers. Taking into account of the fact that the baseline plasma concentrations of IL-1β and IL-1Ra were distinct, the allele-dependent variations followed similar profiles in both B-CLL patients and normal controls. There were no significant differences in IL-6 plasma levels of subjects with different alleles of the IL-6 gene.

Table 2. Allelic distribution and frequencies in B-CLL patients (n = 35) and in normal controls (n = 400). Numbers in parentheses are percentages of cases.

Allele	*2/2*	*_*	*1/1*	*Fq 1*	*Fq 2*	*2-carrier*
IL-1α						
CLL	1 (2.9)	18 (51)	16 (46)	0.714	0.286	19 (54)
Control	32 (8.0)	201 (50)	167 (42)	0.669	0.331	233 (58)
IL-1β (-511)						
CLL	4 (11)	18 (51)	13 (37)	0.629	0.371	22 (63)
Control	72 (18)	182 (46)	146 (36)	0.593	0.408	254 (64)
IL-1β (+3953)						
CLL	0	17 (49)	18 (51)	0.757	0.242	17 (49)
Control	30 (7.5)	164 (41)	206 (52)	0.720	0.280	194 (49)
IL-1Ra*						
CLL	2 (5.7)	15 (43)	16 (46)	0.671	0.271	17 (49)
Control	37 (9.0)	156 (39)	199 (50)	0.701	0.289	193 (49)
IL-6†						
CLL	8 (23)	13 (37)	14 (40)	0.414	0.586	21 (60)
Control	81 (20)	201 (50)	118 (30)	0.454	0.546	282 (71)

Allele x/3 in B-CLL patients and controls: differences not significant (in B-CLL: n = 2, Fq 0.029; in controls: n = 8, Fq 0.100). †Allele G at position –174 of the IL-6 gene is referred to here as allele 2 and allele C at position –174 of the IL-6 gene as allele 1.

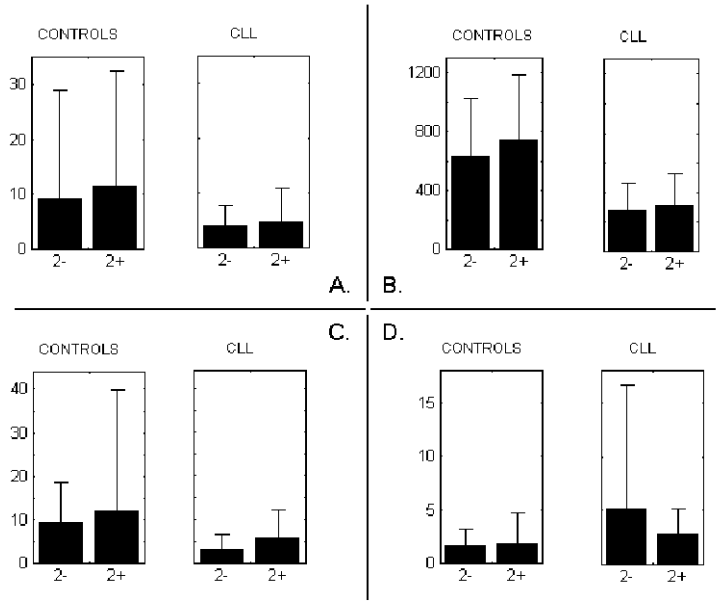

Figure 5. Allele 2 carrier state and cytokine levels among normal controls and B-CLL patients. Panel A: IL-1β (-511) allele 2 carrier state and IL-1β plasma levels. Panel B: IL-1Ra allele 2 carrier state and IL-1Ra plasma levels. Panel C: IL-1β (+3953) allele 2 carrier state and IL-1β plasma levels. Panel D: IL-6 allele 2 carrier state and IL-6 plasma levels. Ordinate: Cytokine concentrations in picograms per milliliter; abscissa: allelic status.

Discussion

In this study we found that the baseline plasma concentrations of IL-1β and IL-1Ra are diminished in B-CLL and plasma IL-6 elevated compared to levels of normal controls. We also found that the levels of IL-1β varied between patients having distinct immunophenotype score. Plasma IL-1β was higher and IL-1Ra/IL-1β ratio lower in patients having non-progressive disease compared with patients representing progressive disease forms. Plasma IL-6 was higher in patients with Binet C disease or anemia, and correlated negatively with hemoglobin levels and positively with ESRs in B-CLL patients. Moreover, we analyzed several polymorphisms of relevant cytokine genes and found that allelic imbalance does not explain the aberrant cytokine levels in B-CLL.

Recent data have shown that expansion of a neoplastic B cell clone in B-CLL is caused primarily by accumulation of cells as a result of the inhibition of apoptosis rather than via straightforward proliferation.[20] Several cytokines have been suggested to regulate apoptosis in B-CLL (see the introduction). IL-1 is known to have indirect hematopoietic activity by inducing gene expression of colony-stimulating factors and IL-6.[21] It has been suggested that the circulating levels of IL-6 represent the best marker of biologically active IL-1.[5]

IL-1β is synthesized as an immature pro-IL-1β form and converted to mature IL-1β after cleavage by interleukin-1 converting enzyme (ICE).[22] Secretion of mature IL-1β is inhibited in the presence of ICE-inhibitor.[5,22] Recent reports concerning the mechanisms of cell death suggest that Fas-mediated apoptosis is dependent on ICE-like protease activity.[23,24] The inhibitors of ICE also inhibit spontaneous and glucocorticoid-induced apoptosis in B-CLL cells.[25,26] As shown here, in B-CLL patients, IL-1β plasma levels are below normal. On this basis one may speculate that the low IL-1β levels observed in B-CLL patients are caused by low ICE activity, especially in patients having more progressive forms of the disease. If so, the protease-mediated cell death mechanisms would not work properly and fast accumulation of malignant cells could occur. This hypothesis is interesting, as the ICE gene is located at chromosome region 11q22.2-q22.3, which is frequently affected in B-CLL.[2,27-29] These factors related to IL-1b secretion and apoptosis could explain the imbalance of IL-1Ra and IL-1β in patients with slowly progressive forms of the disease.

We also consider that the elevated IL-6 plasma levels could be a consequence of low IL-1Ra/IL-1β ratios which lead to high IL-1 activity in B-CLL patients. These pro-inflammatory IL-1Ra/IL-1β ratios and high IL-6 plasma levels could partly contribute to autoimmune phenomena associated with the pathogenesis of B-CLL. In our study the plasma levels of IL-6 were higher among patients with more advanced stages of the disease, in agreement with earlier results of Reittie *et al*.[4] On the other hand, circulating IL-6 has been suggested to be elevated in hairy cell leukemia and non-Hodgkin's lymphoma but not in CLL.[30] In this earlier study, however, only 6 patients with CLL were studied and no disease characteristics were provided.

Furthermore, we found that IL-6 plasma levels were related to hemoglobin levels, to the occurrence of anaemia and to ESRs in B-CLL patients. Phase II trials on cancer patients have shown, that recombinant IL-6 administered intravenously causes transient decrease of hemoglobin levels by way of a hemodilution mechanism and induction of acute phase proteins in the liver.[31,32] A similar biological effect could explain our findings in B-CLL.

In this study we also found that the B-CLL patients with low immunophenotypic scores had higher IL-1β plasma levels than patients with higher scores. To

some extent the immunophenotypic scoring system predicts atypical cell morphology in blood films.[14] These atypical cases of B-CLL have been shown to have distinct immunophenotypic, cytogenetic, morphological and prognostic profiles.[33] Our findings suggest that the atypical forms of B-CLL could also be immunologically divergent from the typical forms of the disease. On the other hand, no systematic study concerning the immunophenotype score and its clinical implications on immune responses or prognosis in B-CLL has been done. Therefore the clinical relevance of this finding have to be confirmed on a larger number of patients.

Within the limits of this study it is impossible to say which are the mechanisms causing the imbalanced cytokine pattern seen in B-CLL. As no allelic imbalance was found, the biological system behind the distinct cytokine profiles observed seems to be more complicated. Further functional studies (concerning eg. ICE) are needed to elucidate disorders in the pathways for cytokine regulation in B-CLL.

Potential implications for clinical practice

- The high plasma IL-1 beta and low plasma IL-1Ra/IL-1β ratio in non-progressive B-CLL suggest that plasma IL-1β, or mechanisms involved in IL-1β secretion may have a protective role in B-CLL.
- The results suggest potential tools for identifying prognostically diverse subgroups or atypical cases of B-CLL.
- The findings exclude the possibility that genetic polymorphism of IL-1α, IL-1β, IL-1 receptor antagonist and IL-6 genes could be used in the risk-assessment of B-CLL.

Contributions and Acknowledgments

All investigators contributed to the design of the study. JH collected data, was responsible for the laboratory analysis of samples, did the statistical analysis of the data, and was principally responsible for writing the paper. JV co-ordinated the study and revised it critically for important intellectual content. LV collected the clinical and DNA samples for the study. TK collected the clinical database for the patients and maintained and updated it throughout the project. MH coordinated the study, revised it critically and provided supervision for the study. All investigators approved the final version of the paper. The order in which the authors' names appear reflects their contribution to the study. We thank Sinikka Repo-Koskinen, Merja Suoranta and Leena Pankko for their technical assistance. We thank the Tampere CLL Group for collaboration.

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Disclosures

Conflict of interest: none.

Reduntant publications: <50%. Part of the data concerning the effect of IL-1 complex gene polymorphisms on plasma IL-1Ra levels in normal controls were published recently (Ref. #8). No data concerning these, or IL-6 polymorphism in B-CLL patients have been published.

Manuscript processing

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