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Serum Levels of Soluble CD8 are Increased in Patients with B Chronic Lymphocytic Leukemia

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Abstract—*Serum levels of the soluble form of the CD8 (s-CD8) antigen were evaluated in the peripheral blood of 44 patients with B-CLL, using an enzyme-linked immunosorbent assay, and were correlated with clinical features and relevant hematological and immunological data. Increased values were observed with respect to normal age-matched controls (mean ± S.E.M. 603 U/ml ± 81 vs. 315 U/ml ± 31, respectively; $P < 0.0001$). This increase was observed in all stages of the disease, excluding stage 0 (mean 277 U/ml ± 45). A general trend pointing to lower values overall in patients with less invasive disease was observed. In fact, the rank correlation test showed that the serum levels of s-CD8 correlate significantly with the WBC counts, the CD4/CD8 ratio, and also with the levels of serum immunoglobulins. On the contrary, no correlation was observed between s-CD8 levels and the absolute number of circulating CD8+ cells in individual cases. Therefore, the increase of s-CD8 is unlikely to be a mere expression of the increase of the CD8 cell number, but seems related to an increase of the activation phenomena involving the CD8+ T cell subset.*

INTRODUCTION

B-CELL chronic lymphocytic leukemia (B-CLL) is a lymphoproliferative disorder characterized by the accumulation of relatively mature monoclonal B lymphocytes in the peripheral blood, bone marrow and other involved tissues. The residual T cell population in this disease does not belong to the neoplastic clone, as indicated by the lack of chromosome abnormalities and by the heterozygosity for glucose-6-phosphate dehydrogenase [1, 2]. Nevertheless, a number of phenotypic and functional abnormalities of T cells have been described in B-CLL [3–11]. In particular, increased absolute numbers of circulating CD8+ lymphocytes associated with heightened *in vitro* suppressor functions have been described [3–5], with a reversed CD4/CD8 ratio which has been claimed to correlate with the progression of the disease [9–11]. However, this finding has been a matter of intense debate, especially in terms of correlation with the disease stages [12, 13].

The CD8 molecule is one of the various cell surface antigens involved in T cell functions. It exists in homomultimeric configurations on the membrane of human T cells, the most common being homodimers of 52, 67 and 76 kD [14]. In a recent study, Fujimoto *et al.* [15] have provided evidence that, like other surface molecules [16, 17], there is a physiological release of a cell-free soluble form of the CD8 antigen (s-CD8) from CD8-bearing lymphocytes. In the present study we determined the levels of s-CD8 in the serum of 44 patients with B-CLL looking for a relationship with a number of clinico-hematological parameters.

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MATERIALS AND METHODS

Patients

The study included 44 patients with B-CLL, 27 men and 17 women, with a mean age of 62 years. The diagnosis was made on the basis of established hematological criteria and confirmed in all cases by phenotypic analyses on peripheral blood (PB) cell suspensions. Patients were graded according to Rai's staging system [18], as follows: 13 stage 0, 11 stage I, 11 stage II, and nine stage III plus IV. Informed consent was obtained from all patients before the study. The total lymphocyte count ranged from 11.6×10^9/l to 144×10^9/l. Thirty-three patients never received treatment, while 11 patients had been previously treated with low doses of alky-

lating agents but at the time of this study they had been off treatment for at least 15 weeks. The mean serum immunoglobulin levels, as determined by radial immunodiffusion, were: IgG 1026 mg/ml ± 136 (control range: 800–1680 mg/ml); IgA 150 mg/ml ± 24 (control range: 60–400 mg/ml); IgM 100 mg/ml ± 12 (control range: 50–250 mg/ml).

Fifty-six healthy volunteers in the same age range were used as controls.

Detection of soluble CD8 molecule

Soluble CD8 levels in the serum of B-CLL patients and of normal controls were determined using a sandwich enzyme immunoassay (T8 CELLFREE, T Cell Sciences, Inc., Cambridge, Mass.) which used two monoclonal antibodies (MoAbs) recognizing different epitopes of the CD8 molecule. The method has been described by Brown *et al.* [19]. Briefly, the s-CD8 molecule available in the test samples or in the standards binds to the polystyrene microtiter wells previously incubated with an anti-CD8 (C9) coating MoAb (100 μl/well of a solution of 0.1 ml of anti-CD8 in 10 ml PBS containing buffer). A horseradish peroxidase-conjugated anti-CD8 MoAb (B12) directed against a second epitope of the CD8 molecule binds to the s-CD8 captured by the first antibody and completes the sandwich. After washing to remove the unbound enzyme-conjugated anti-CD8 MoAb, a substrate solution is added to the wells. The reaction is then stopped and the absorbance determined at 490 nm. A standard curve is prepared by using different reference preparations containing known concentrations of s-CD8 expressed as arbitrary units per milliliter. Results were expressed as total amount of s-CD8 Units per milliliter of serum.

Surface marker analysis

Mononuclear cells were separated by Ficoll-Hypaque (F/H) density gradient from freshly drawn heparinized PB and washed three times in sterile phosphate-buffered saline (PBS). Adherent cells were removed following incubation at 37°C for 45 min in plastic Petri dishes. The viability, assessed by the trypan blue exclusion test was always greater than 95%.

The following MoAbs, designated according to the Oxford workshop [20], were used to characterize the phenotype of enriched T cell populations. OKT3 (CD3) MoAb (Ortho Pharmaceuticals, NJ) defines T cells; OKT4 (CD4) positive cells include helper/inducer lymphocytes while cells reactive with OKT8 (CD8) MoAb include cytotoxic/suppressor cells.

The frequency of lymphocytes positive for the above quoted reagents was determined by flow cytometry, using a flow cytometer (Cytofluorograph IIs Ortho) equipped with Argon laser and operating at a wavelength of 488 nm to excite fluorescein (FITC) and phycoerythrin (PE). Forward angle and 90° scatter were used to discriminate viable lymphocytes from dead cells and monocytes. FITC and PE mouse immunoglobulins of the same isotype (IgG_1, IgG_{2a+b}) were used as negative controls and at least 10,000 cells were scored. Double staining with CD4 PE-conjugated or CD8 PE-conjugated MoAbs was used to investigate the coexpression of these antigens on the same cell.

Analysis of data

All data are presented as means ± standard error (S.E.M.s) and comparisons between values were carried out using either the Cockran–Cox test or the Spearman's rank correlation test. A P value < 0.05 was considered significant.

RESULTS

As shown in Fig. 1 the mean value of s-CD8 in the serum of patients with B-CLL taken as a group was statistically higher than in age-matched controls (603 U/ml ± 81 vs. 315 U/ml ± 31, $P < 0.001$). Except stage O B-CLL, in only eight out of 31 cases the s-CD8 levels fell within the normal range. No statistically significant difference was observed between the values detected in untreated patients and cases who had been previously treated.

According to different stages of the disease (Table 1), no difference was observed between stage 0 B-CLL patients and controls (277 U/ml ± 45 and 315 U/ml ± 31, respectively; P: ns). On the other hand, a statistically significant difference was found between the levels of s-CD8 in stage 0 and

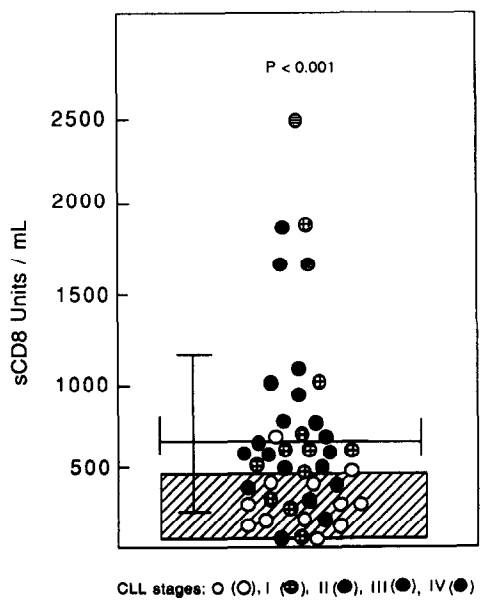

Fig. 1. Serum levels of s-CD8 in B-CLL patients (subdivided according to stages) and in healthy controls.

Table 1. Clinical findings, hematological parameters and s-CD8 levels in B-CLL patients (mean ± S.E.)

Group		n	WBC/cumm	Lymphocytes/cumm	CD4/CD8 ratio	CD8 cells/cumm	s-CD8 values μ/ml
B-CLL	(as a group)	44	45,665 ± 6468	38,156 ± 5730	1.51 ± 0.16	1231 ± 380	603 ± 81
B-CLL stage	0	13	17,376 ± 2348	11,393 ± 2083	2.16 ± 0.29	719 ± 1,290	277 ± 45
	I	11	42,300 ± 11,039	37,033 ± 11,055	1.23 ± 0.30	884 ± 146	640 ± 147
	II	11	49,311 ± 10,800	43,144 ± 10,341	1.15 ± 0.16	850 ± 166	839 ± 98
	III plus IV	9	86,023 ± 19,246	71,190 ± 16,815	0.89 ± 0.12	2376 ± 1,133	762 ± 185
Controls		20	5236 ± 315	1697 ± 182	1.89 ± 0.2	436 ± 56	315 ± 31
B-CLL as a group vs. controls			< 0.001	< 0.001	n.s.	< 0.01	< 0.001
B-CLL stage 0 vs. stage I			< 0.05	< 0.05	< 0.05	n.s.	< 0.05
B-CLL stage 0 vs. stage II			< 0.01	< 0.01	< 0.01	n.s.	< 0.05
B-CLL stage 0 vs. stage III plus IV			< 0.005	< 0.005	< 0.001	n.s.	< 0.02
B-CLL stage I vs. stage C			< 0.005	< 0.01	< 0.05	< 0.01	< 0.05
B-CLL stage II vs. stage C			< 0.001	< 0.001	< 0.005	< 0.02	< 0.02
B-CLL stage III plus IV vs. C			< 0.001	< 0.001	< 0.001	n.s.	< 0.02

all other stages, but no differences were observed between stages I, II, and III plus IV. All values in these groups of patients are statistically significant with respect to controls ($P < 0.05$, < 0.02, < 0.02 in stages I, II and III plus IV, respectively).

The levels of s-CD8 were also correlated with the number of WBC/ml, with CD4/CD8 ratio and with immunoglobulin levels (Table 2). A general trend pointing to overall lower values in patients with less invasive disease was observed. In fact, the rank correlation test, determined in all patients taken as a group, showed a mild direct correlation between serum levels of s-CD8 and WBC counts and a moderate indirect correlation with the CD4/CD8 ratio and with the levels of serum immunoglobulins. These analyses were also performed after excluding the 11 previously treated patients but no differences have been detected with respect to the correlations observed using the entire population.

No correlation (r_s: 0.01; P: ns) was found between s-CD8 levels and the absolute numbers of CD8 circulating lymphocytes. The lack of such a correlation has also been observed in controls (r_s: 0.22; P: ns).

DISCUSSION

Our study demonstrates that serum levels of s-CD8 are increased in patients with B-CLL, as

Table 2. s-CD8 values in different groups of B-CLL patients subdivided according to different hematological parameters

Different groups of B-CLL patients	n	s-CD8 values* (U/ml)	r_s†
Patients with WBC (× 10^9/l)			
< 25	19	381 ± 54	
25–49	11	667 ± 179	0.32 ($P < 0.05$)
50–75	5	1180 ± 361	n 44‡
> 75	9	721 ± 144	
Patients with CD4/CD8 ratio			
> 1.4	13	260 ± 46	
1.4–0.8	9	662 ± 158	0.50 ($P < 0.01$)
> 0.8	5	641 ± 244	n 27‡
Patients with serum 1 g (mg/100 ml)			
> 15	12	423 ± 100	
15–18	26	531 ± 99	0.41 ($P < 0.01$)
< 8	6	662 ± 232	n 44‡

*Mean ± S.E. The mean values among between different groups were not statistically significant.

†Correlation between s-CD8 levels and different hematological parameters.

‡All patients taken as a group.

compared to normal, with the only exception being patients with early disease (stage 0). Although the increase is not closely associated to other clinical features, a general trend pointing to overall lower values in patients with less invasive disease was observed.

A possible interpretation for the increased s-CD8 values found in B-CLL is likely to be linked with the extension and/or the functional status of the CD8+ cell compartment. Our data suggest that the explanation does not simply rest with the increase of circulating CD8+ cells observed in B-CLL [7–11], since no correlation could be found, either in this disease or in controls, between s-CD8 levels and circulating CD8+ cells. On the other hand, a possible organ redistribution of CD8+ cells, with consequent margination from the peripheral blood of an unknown and variable number of the CD8+ population, is unlikely to occur in B-CLL, as suggested by a previous report [6]. Although the immunological role of s-CD8 molecule and the exact mechanisms regulating its cellular release are not yet established, current concepts view the release of the s-CD8 molecule as the result of an activation of CD8+ lymphocytes. In fact, both cellular stimulation *in vitro* induces the release of heightened amounts of s-CD8 molecule [15] and immune activation *in vivo* leads to the increased serum s-CD8 levels [15, 18, 19].

Studies on the source of s-CD8 pointed out that a CD8-like protein of 27,000 MW (smaller than its membrane counterpart) is preferentially released by a subpopulation of antigen-specific CD8 lymphocytes bearing the CD8+/HLA−DR+ phenotype [15, 19, 21]. According to this interpretation, high levels of this soluble antigen have been observed in the sera of patients with acute infectious mononucleosis [21], a disease characterized by an activation of cytotoxic CD8+ cells. A possible increase of T-cell activation in B-CLL could be related to the presence of a cytotoxic effector cell population arising in response to the yet unknown causative agent of B-CLL, or to its associated neoantigens. In this regard, an expansion of the cytotoxic CD8 cellular compartment has been hypothesized in the peripheral blood of patients with B-CLL [22]. High serum levels of s-CD8 antigen have been recently described in other lymphoid malignancies, i.e. in children with acute lymphoblastic leukemia and Hodgkin's or non-Hodgkin's lymphomas [23, 24], and AIDS-related disorders [25].

The evidence, herein demonstrated by the rank correlation test, that a mild correlation exists between the serum levels of s-CD8 and the WBC counts, the CD4/CD8 ratio, and the levels of serum immunoglobulins supports the role that CD8+ cells play in this disorder, pointing to the fact that the state of activation of CD8+ cells correlates with the progression of the disease [9–11], including their relationship with the degree of hypogammaglobulinemia [8]. We also correlated the serum levels of sCD8 with serum soluble IL-2 receptor levels in the same series of patients but we were unable to demonstrate any statistically significant correlation between the two parameters (data not shown).

As far as the mechanism of CD8 release is concerned, it has been demonstrated that the release of cell surface antigen after antibody treatment is not the result of cell destruction, but is an antigen-specific phenomenon since the release of Leu-1 determinant after anti-Leu-1 antibody administration is not accompanied by the release of Leu-2 or Leu-3 antigens [15]. At least two possibilities account for the CD8 release: shedding from the cell surface and secretion by exocytosis. Further studies evaluating cell lines or mutant cells lacking CD8 antigen expression on the surface, internal labeling, and pulse and chase experiments, may resolve these questions. Furthermore, a more precise definition of the function of cell subsets involved in the process of shedding the soluble CD8 antigen and of its role in health and disease will contribute to the comprehension of the relevance of the CD8 molecule in intercellular communications. This will further clarify the function of CD8 subpopulation in B-CLL patients, thus generating new insights into the biological workings of the immune system in B-CLL.

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