

ABNORMAL DISTRIBUTION OF CD8 SUBPOPULATION IN B-CHRONIC LYMPHOCYTIC LEUKEMIA IDENTIFIED BY FLOW CYTOMETRY

L. W. M. M. TERSTAPPEN,* B. G. DE GROOTH,* W. VAN BERKEL,†
C. H. H. TEN NAPEL† and J. GREVE*

*Twente University of Technology, Department of Applied Physics, Cell Characterization Group,
P.O. Box 217, 7500 AE Enschede and †Ziekenzorg Hospital, Department of Internal Medicine,
Haaksbergerstraat 55, 7513 ER Enschede, The Netherlands

(Received 1 June 1987. Revision accepted 26 March 1988)

Abstract—We studied the occurrence of T-cell subpopulations for patients with B-cell chronic lymphocytic leukemia. The CD8⁺ population was divided into CD8⁺ suppressor (CD8a⁺) and CD8⁺ cytotoxic (CD8b⁺) lymphocytes using difference in orthogonal light scattering.

Average CD4⁺/CD8⁺ ratios determined for all patients were decreased. For individual patients this sometimes was not true. In contrast CD4⁺/CD8a⁺ ratios were markedly increased in all individual patients. The CD8⁺ lymphocytes appeared to consist mainly of CD8b⁺ lymphocytes. Moreover the CD8b⁺/CD8⁺ ratio correlated with clinical stage: untreated patients (stage 0 of Rai) have smaller CD8b⁺/CD8⁺ ratios than patients with advanced stages of Rai.

Key words: B-chronic lymphocytic leukemia (B-CLL), leukemia, white blood cells, leukocytes, suppressor lymphocytes, cytotoxic lymphocytes.

INTRODUCTION

VARIOUS T-cell abnormalities have been reported for B-chronic lymphocytic leukemia (B-CLL) patients. Most authors describe an increase in CD8 positive lymphocytes (CD8⁺) and a decrease in CD4 positive lymphocytes (CD4⁺) [1-8]. Functional T-cell studies yield contradictory results; normal [9] or decreased helper function is described [10-12] as well as decreased [13] or increased suppressor function [14, 15].

We have shown previously that using flow cytometry (FCM) two populations of lymphocytes can be distinguished by measuring the light scattering signal in the orthogonal direction [16]. It was demonstrated that these subpopulations consist of regulatory and B-lymphocytes (low orthogonal light scattering signal) and cytotoxic lymphocytes (high orthogonal light scattering signal). In another report we have shown that the CD8⁺ lymphocyte population could be divided in a suppressor- and a cytotoxic lymphocyte CD8⁺ subpopulation [17].

In this study we have used FCM to get more detailed insight in the occurrence of CD8⁺ suppressor

and CD8⁺ cytotoxic lymphocytes in peripheral blood of B-CLL patients.

MATERIALS AND METHODS

Patient populations

The study included 22 patients with B-CLL with a mean age of 59 (range 41-85). The patients were graded using the Rai clinical staging [18]. Seven patients with stage 0 with no progression for at least two years were included, one of them was previously treated because of severe disease (IV Rai). Four patients graded stage 0 in partial remission, all previously stage IV and successfully treated with chlorambucil sometimes in combination with prednisolone. These patients received maintenance treatment. Also included were four patients graded stage IV, two patients with stage III and one patient with stage II before receiving any treatment and four patients graded stage IV showing no‡ or insufficient§ improvement after treatment of at least one year with chlorambucil sometimes in combination with prednisolone.

Preparation of lymphocytes

Human blood was collected between 8 and 9 p.m. by venipuncture into vacutainer tubes containing heparin as anticoagulant (150 USP U sodium heparine/10 ml Veno-

Abbreviations: B-CLL, B-cell chronic lymphocytic leukemia; FCM, flow cytometer; FITC-GAM, fluorescein isothiocyanate labeled goat antimouse immunoglobulin.

Correspondence to: Dr J. Greve, University of Twente, Dept of Applied Physics, Cell Characterization Group, P.O. Box 217, 7500 AE Enschede, The Netherlands.

‡No improvement is defined as no improvement of white blood and thrombocyte count as well as hemoglobin concentration.

§Insufficient improvement is defined as a decrease in white blood cell count but no improvement of thrombocyte count and hemoglobin concentration.

ject Terumo Europe NV). Lymphocytes were obtained after density separation as described in detail elsewhere [17]. Lymphocytes were adjusted to a concentration of 1.10^7 lymphocytes per ml with a standard buffer (PBS containing 0.05% sodium azide and 1% bovine serum albumin (BSA)).

Immunofluorescence

The following monoclonal antibodies (Mabs) were used: (a) *unlabelled Mabs*: CD8 (anti-leu2a, from Becton Dickinson, Amersfoort, The Netherlands) T suppressor-cytotoxic marker; CD4 (RIV 6, from National Institute for Public Health and Milieu (RIVM), Rijswijk, The Netherlands) T helper-inducer marker; CD3 (RIV 3, from RIVM) pan T-cell marker; (b) *fluorescein (FITC) labeled Mabs*: CD3 (anti-leu4, from Becton Dickinson), CD4 (anti-leu3a, from Becton Dickinson); (c) *phycoerythrin (PE) labeled Mab*: CD8 (anti-leu2a, from Becton Dickinson). For second step reagents FITC conjugated goat antimouse immunoglobulin (FITC GAM) (Central Laboratory of Blood Transfusion Service (CLB) Amsterdam, The Netherlands) was used. All reagents were pretitered by

using normal lymphocytes to determine optimal conditions for binding. One hundred microliters of cell suspension was incubated with 20 μ l diluted Mab. Mabs were diluted in standard buffer for 30 min at room temperature. For indirect fluorescence the cells were washed twice with standard buffer and resuspended in 100 μ l diluted second step reagent. The cells were incubated for 30 min at room temperature. After incubation the cells were washed twice and resuspended in 1-ml standard buffer and kept on ice until use.

Flow cytometric analysis was performed the same day to preserve both optimal binding of monoclonal antibody and light scatter properties of lymphocytes [19, 20].

Flow cytometry measurements

The FCM was described in detail elsewhere [17]. A 3-W argon ion laser tuned at 488 nm operating at a light intensity of 100 mW was used. FITC emission was measured with a six-cavity bandfilter (510–550 nm, Pomfret Research Optics, Inc., Stanford, CT, U.S.A.). The PE emission was measured with an OG 570 colored glass filter (Schott, Tiel, The Netherlands). Reproducible apparatus

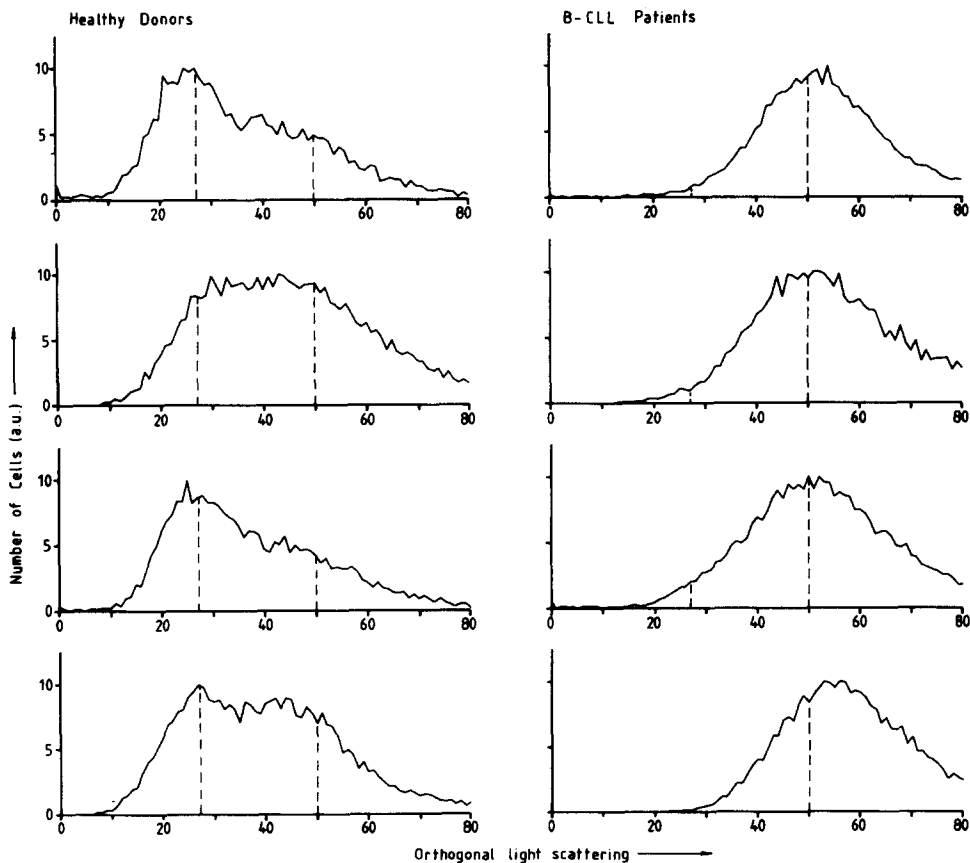


FIG. 1. Orthogonal light scatter histograms of CD8⁺ lymphocytes of four healthy donors (left) and four B-CLL patients (right). Note the two populations in orthogonal light scatter histograms of healthy donors whereas in B-CLL patients the same populations are present; however, the population showing a low orthogonal light scattering intensity is very small. Dashed lines are indicated at the positions of the maxima of the populations.

TABLE 1. RELEVANT CLINICAL DETAILS, PERCENTAGES OF CD3⁺, CD4⁺, CD8⁺ LYMPHOCYTES AND CD8b⁺/CD8⁺ RATIOS OF 22 B-CLL PATIENTS AND 30 HEALTHY CONTROLS

Patient	Sex/age	Rai stage	Treatment	Lymphocyte count $\times 10^6/\text{ml}$	% CD3	% CD4	% CD8	%CD8b/CD8	
1	M85	0	No	81.0	2.4	1.7	2.2	74	
2	F56	0	No	74.6	23.4	8.3	4.4	88	
3	M86	0	No	97.6	3.9	1.8	2.4	79	
4	M77	0	No	13.6	11.1	10.4	2.2	70	
5	M41	0	No	39.0	9.1	7.6	2.7	84	
6	M68	0	No	37.0	7.7	4.9	1.8	87	
7	M54	0	No	5.9	25.9	17.4	8.0	87	
8	M46	0	Yes	2.3	48.3	16.2	31.2	98	
9	F70	0	Yes	3.4	31.8	9.8	19.8	99	
10	M56	0	Yes	15.9	42.0	20.1	18.7	95	
11	M50	0	Yes	16.0	16.7	5.8	5.8	97	
12	M52	II	No	69.6	6.5	3.5	2.5	93	
13	F50	III	No	261.0	1.5	0.7	0.7	94	
14	F59	III	No	257.0	3.6	2.0	1.3	96	
15	M80	IV	No	44.1	15.4	7.3	2.2	95	
16	M47	IV	No	116.0	3.9	2.4	2.0	91	
17	M65	IV	No	185.2	1.5	0.7	0.5	93	
18	M46	IV	No	223.0	7.6	3.1	4.3	94	
19	M66	IV	Yes	1.4	40.0	26.0	15.0	99	
20	M45	IV	Yes	7.4	6.3	3.1	1.1	94	
21	M75	IV	Yes	10.4	25.0	17.5	10.0	93	
22	M62	IV	Yes	130.6	2.2	1.5	0.7	92	
range:									
min.				41	1.4	1.5	0.7	0.5	70
max.				85	261.0	48.3	26.0	31.2	99
mean				76.9	15.3	7.8	6.3	90.5	
\pm S.D.				± 84.7	± 14.5	± 7.2	± 8.0	± 7.8	
30 healthy controls									
range:									
min.				22	1.1	59	38	14	17
max.				85	4.3	86	66	28	69
mean				2.3	69.8	48.9	20.5	39.2	
\pm S.D.				0.7	5.7	6.5	3.8	11.9	

conditions for the measurement of light scatter histograms of lymphocytes were ensured by adjustment of the FCM with standard microspheres. Light scatter histograms of lymphocyte subpopulations were obtained after gating on the fluorescence intensity of immunofluorescently labeled cells. Gates are chosen such that cells are definitely positive: less than 0.01% of the cells of control samples fall within the gates.

RESULTS

Methodology

Choice of stain. The relative number of T lymphocytes in B-CLL patients can be reduced from 60 to 80% of the total number of lymphocytes to 2% or less. In order to obtain reliable data on relative and absolute occurrence of the T-cell subsets amongst all lymphocytes one therefore has to study a large number of cells in a very selective way.

With the monoclonal antibodies used CD3, CD4 and CD8 the selectivity condition is met. In flow cytometry the presence of monoclonal antibodies on cell surfaces is detected with either direct or indirect immunofluorescence staining. We have compared direct and indirect immunofluorescence staining in order to investigate the possibility of detecting positive cells that occur in a frequency below 1%.

When using the direct immunofluorescence labeling, no reliable data can be obtained when positive cells occur in a frequency of 2% or less, since 1.3% of GAM stained lymphocytes are false positive. With direct immunofluorescence labeling using a fluorescently labeled irrelevant antibody (Ig1-FITC) as control, down to 0.05% positive cells can be detected in a reliable way. All further data given in this paper were therefore obtained by using direct immunofluorescence labeling.

CD8a and CD8b determination

Determination of CD8⁺ subpopulations was performed by dividing the orthogonal light scatter histograms into two components with a low (CD8a) and a large (CD8b) orthogonal light scattering signal as described in detail in earlier reports [16, 17]. In B-CLL patients the occurrence of both populations strongly differs from that of healthy donors as is shown in Fig. 1 for four patients and four controls. Dashed lines are indicated at the positions of the maxima of the two populations. In healthy controls two populations can be seen. In B-CLL patients the same populations are present, however, the population showing a low orthogonal light scattering intensity is very small.

Therefore it may even be that for some B-CLL patients only the population with the large orthogonal light scattering appears in the histograms. A large variation in the occurrence of CD8⁺ subpopulations of healthy donors is observed [17]. CD8b⁺/CD8⁺ ratios varied from 17 to 69 (mean 39). For individuals the variation in CD8b⁺/CD8⁺ ratios determined in a time period of 3 months is considerably less. We found a range of CD8b⁺/CD8⁺ ratios for one healthy donor of 24–40 (mean 36, $n = 5$), for one B-CLL patient graded stage 0, a range of 74–79 (mean 76, $n = 3$) was found and for one B-CLL patient graded stage IV a range of 91–99 (mean

95, $n = 5$) was found. No relation between CD8b⁺/CD8⁺ ratio and age could be detected in the control group and the B-CLL patients.

B-CLL patients

Absolute number of T-cell subpopulations. The main clinical details of the patients and the results of the tests are shown in Table 1. The range, mean and standard deviations from the control group are shown at the bottom of the table. Lymphocyte counts for B-CLL patients varied considerably (range 1.4–261 × 10⁶/ml). It is clear that CD3⁺, CD4⁺ and CD8⁺ percentages are lower than in healthy controls which is obviously due to the presence of the large number of B cells. The ratio of CD8b⁺ to CD8⁺ lymphocytes (CD8b⁺/CD8⁺) gives straightforward results: all patients are strongly abnormal compared with healthy donors (see also Fig. 3). For further evaluation of the results the absolute number of CD3⁺, CD4⁺ and CD8⁺ lymphocytes are presented in Fig. 2. Although the mean values of the absolute number of CD3⁺, CD4⁺ and especially CD8⁺ lymphocytes are increased no conclusive proof can be given for individual patients because of the very large patient to patient variation.

Ratios of T-cell subpopulations. CD4⁺/CD8⁺ ratio's were calculated as well as the CD4⁺/CD8a⁺ ratios. They are presented in Fig. 3 together with the

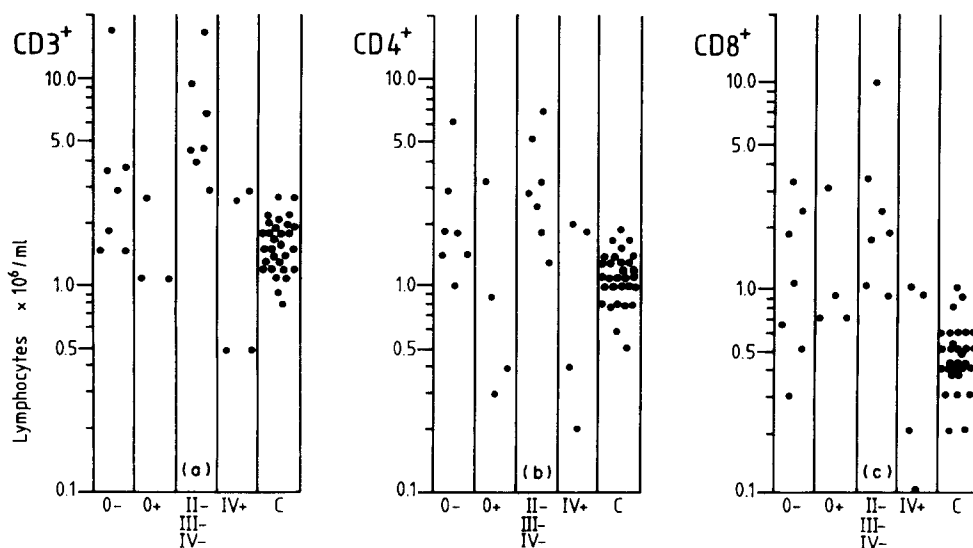


FIG. 2. Absolute number of CD3⁺ (a), CD4⁺ (b) and CD8⁺ lymphocytes (c) of 22 B-CLL patients subdivided into clinical stage by Rai and presence (+) or absence (-) of treatment, compared with 30 healthy individuals (C). Lymphocyte counts are expressed on a logarithmic scale. The Kruskal-Wallis test showed that the data given for patients and healthy individuals are significantly different ((a) $p < 0.001$, (b) $p < 0.005$ and (c) $p < 0.0001$).

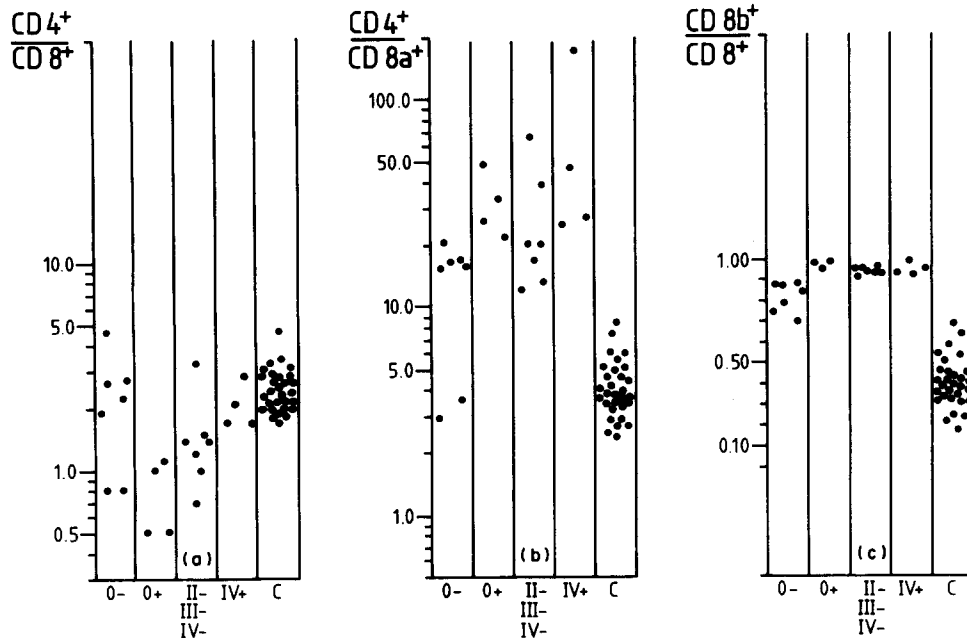


FIG. 3. $CD4^+/CD8^+$ ratios (a), $CD4^+/CD8a^+$ true helper/suppressor ratios (b) and contribution of $CD8^+$ cytotoxic lymphocytes to the $CD8^+$ population $CD8b^+/CD8^+$ (c), of 22 B-CLL patients classified with the clinical staging model of Rai and presence or absence of treatment, compared with 30 healthy individuals (C). Note that the figures are on different scales. The Kruskal-Wallis test showed that the data given for patients and healthy individuals are significantly different ((a) $p < 0.0005$, (b) $p < 0.00001$ and (c) $p < 0.00001$).

ratio of $CD8^+$ cytotoxic lymphocytes to all $CD8^+$ lymphocytes ($CD8b^+/CD8^+$).

Patients were subdivided in early and late disease as well as treated and untreated. The B-CLL group showed decreased mean $CD4^+/CD8^+$ values ($1.7 \pm S.D. 1.0$) compared with healthy donors ($2.5 \pm S.D. 0.6$) but a considerable number of individual $CD4^+/CD8^+$ ratios fall within the normal range. When subdivided into clinical stages, stage IV patients on therapy all fall within the normal range whereas all treated stage 0 patients showed a significantly decreased $CD4^+/CD8^+$ ratio (Fig. 3a). When the $CD4^+/CD8a^+$ ratios are determined a completely new perspective is gained (Fig. 3b). $CD4^+/CD8a^+$ ratios are strongly increased in the B-CLL group also when considered individually. Exceptions are the untreated stage 0 patients where two out of seven patients showed values overlapping with the normal individuals. Note the logarithmical scale. In Fig. 3c $CD8b^+/CD8^+$ ratios are given. All patients showed an increase in $CD8b^+/CD8^+$ compared with the control group. It is remarkable that untreated stage 0 CLL patients showed a smaller increase than the other patients.

DISCUSSION

The low frequency of T lymphocytes in B-CLL patients makes it very difficult to determine relative and absolute numbers of T-cell subpopulations. In this study we demonstrate that using a flow cytometer, lymphocytes directly immunofluorescently labeled with monoclonal antibodies can be detected reliably even if they occur in a frequency down to 0.05%.

Most studies on T-cell subpopulations in B-CLL patients use T-cell enrichment by E-rosette formation and subsequent density separation for determination of T-cell subpopulations in B-CLL patients. Using this technique no accurate information on T-cell subpopulations is obtained because of variation in affinity during E-rosette formation, limited purity or enriched T cell and selective loss of T-cell subpopulations by enrichment [21]. Further complications arise from E-rosette formation by immunoglobulin positive cells [22] and presence of E receptors in B-CLL cells [23]. To our knowledge only three studies have been reported in which direct assessment of T-cell subpopulations in B-CLL is per-

formed [2, 5, 8]. In these studies the median of the absolute number of T cells was enlarged especially of CD8⁺ cells. Our results are in agreement with these studies (Fig. 2). However, a very large patient to patient variation is observed in absolute number of CD3⁺, CD4⁺ and CD8⁺ lymphocytes. Absolute number of T cells can be increased up to 17×10^6 /ml.

This could suggest monoclonal proliferation of T cells alongside the proliferating B cells. This is not the case however, because different T cell subsets, i.e. CD4⁺, CD8⁺, account for the increase.

Concomitant with other reported studies [1–6] the mean CD4⁺/CD8⁺ ratios in B-CLL patients is decreased (Fig. 3). However, it is not possible to draw conclusions from an individual CD4⁺/CD8⁺ ratio measured for a patient, since substantial overlap exists between CD4⁺/CD8⁺ ratios of controls and patients. No obvious relation with advancing disease as is reported by some authors [1, 3] could be found, except for patients showing no or insufficient improvement on treatment which all showed normal CD4⁺/CD8⁺ ratios.

The CD4⁺/CD8⁺ ratio is often presented as helper/suppressor ratio. However, using this definition heterogeneity of CD8⁺ lymphocytes is not taken in consideration. In this study we have shown that with B-CLL patients this leads to completely wrong conclusions as becomes clear when CD4⁺/CD8a ratios are determined. This ratio more closely represents the helper/suppressor ratios [17]. The ratios are all strongly increased which suggest a relative increase of helper lymphocytes instead of the assumed increase of suppressor lymphocytes. Only two patients with benign disease showed a normal CD4/CD8a ratio whereas for the other five patients the relative increase was less as compared with the other groups (Fig. 3b).

With the determination of the CD4⁺/CD8b⁺ ratios a clear new view on T-cell subsets in B-CLL is revealed. No increase of suppressor lymphocytes is evoked by the proliferating B lymphocytes as is suggested by others [3], but an increase of CD8⁺ cytotoxic lymphocytes. This is clearly demonstrated by the determination of the contribution of CD8⁺ cytotoxic lymphocytes to the CD8⁺ population (CD8b⁺/CD8⁺) which is strongly increased in all B-CLL patients (Fig. 3c). Since the absolute number of CD8⁺ lymphocytes was not decreased in B-CLL patients (Fig. 2c), the increase of CD8b⁺/CD8⁺ is due to an increase of the absolute number of CD8b⁺ lymphocytes. An obvious relation with advancement of disease is found: all untreated stage 0 patients showed significantly less increased CD8b⁺/CD8⁺ ratio than patients on therapy and patients with

advancing disease. The CD8b⁺/CD8⁺ may be a parameter which can give an indication on prognosis or the results of treatment. (This is suggested by the well-known fact that prognosis of treated patients in partial remission is worse as compared with untreated stage 0 patients.) In agreement with this, one patient initially treated but in remission for three years had also a less increased CD8b⁺/CD8⁺ ratio (patient 7, Table 1). The suggestion that increase in CD8⁺ or T γ ⁺ lymphocytes in B-CLL patients [1] is doubtful, since we have shown that this increase is merely due to increase of CD8⁺ cytotoxic lymphocytes. An attractive alternative hypothesis is that the hypogammaglobulinaemia is due to a decrease of normal B lymphocytes.

Support for an important influence of cytotoxic lymphocytes in B-CLL patients is given by studies where a significant increase of HNK-1 (leu7) lymphocytes is found in B-CLL [8, 24]. Studies on helper and suppressor function in B-CLL patients give contradicting results. Increased [14, 15] or decreased [13] suppressor as well as increased [10–12] or normal [9] helper function are described. These contradictions could be caused by different methodology. It has to be noted that we have demonstrated that the CD8b⁺ lymphocytes in B-CLL patients have the physical properties and membrane receptor characteristics of cytotoxic lymphocytes. Our results suggest that functional studies of cytotoxic lymphocytes in B-CLL patients may be of great importance. The functional properties of these lymphocytes are still under investigation. An interesting hypothesis is that the number of cytotoxic lymphocytes is increased in B-CLL patients because the immune system is involved in an active battle against the leukemic cells. If it is assumed that these cytotoxic cells are made because they are directed specifically against leukemic cells, the decreased cytotoxicity found in NK cell assays and antibody dependent cytotoxicity mediated assays in B-CLL patients is explained since in these assays the cytotoxicity for other cell types is tested [24–26]. If these hypothesis are true new prospectives for the understanding and treatment of leukemic diseases are revealed.

Acknowledgements—We are grateful to Y. Kraan and I. Segers for skillful laboratory work and to Dr R. Holdrinet (Radboud ziekenhuis Nijmegen, The Netherlands) and Dr G. S. Hoogendoorn (St Geertruiden ziekenhuis, Deventer, The Netherlands) for letting us study their patients (patients 12, 14; Table 1, respectively). We thank Dr D. Catovsky (Hammersmith Hospital, London, U.K.) for his critical review of this manuscript.

REFERENCES

1. Catovsky D., Lauria F., Matutes E., Foa R., Mantovani V., Tura S. & Galton G. A. G. (1981) Increase in CD4 lymphocytes in B-cell chronic lymphocytic leukemia. *Br. J. Haemat.* **47**, 539.
2. Mankey S. M., Alexander H. D., Agnew A. N. D., McConnell R. E., Morris T. C. M., Robertson J. H., Crockard A. D. & Buidges J. M. (1986) Enumeration of absolute numbers of T-lymphocyte subsets in B-chronic lymphocytic leukemia using an immunoperoxidase technique: relation to clinical stage. *Br. J. Haemat.* **62**, 257.
3. Heurmann F., Lochner A., Philippen H., Janer B. & Ruhl H. (1982) Imbalance of T-cell subpopulations in patients with chronic lymphocytic leukemia of the B-cell type. *Clin. exp. Immun.* **49**, 157.
4. Mills K. H. G. & Cawley J. C. (1982) Suppressor T cells in B-cell chronic lymphocytic leukemia: relationship to clinical stage. *Leukemia Res.* **6**, 653.
5. Mittelman A., Denny T., Gebhard C., Cirincione C., Kurland E. & Kozinek B. (1984) Analysis of T cell subsets in B-cell chronic lymphocytic leukemia: a correlation with the stage of disease. *Am. J. Haemat.* **16**, 67.
6. Platsoucas C. D., Gatinski M., Kempin S., Reich L., Clarkson B. & Good R. A. (1982) Abnormal T-lymphocyte subpopulations in patients with B-cell chronic lymphocytic leukemia: an analysis by monoclonal antibodies. *J. Immun.* **129**, 2305.
7. Kay N. E. & Kaplan M. E. (1984) Defective expression of T-cell antigens in chronic lymphocyte leukemia: relationship to T-cell dysfunction. *Br. J. Haemat.* **57**, 105.
8. Velardi A., Prchal J. T., Prasthofer E. F. & Grossi C. E. (1985) Expression of NK-lineage markers on peripheral blood lymphocytes with T-helper (leu3⁺/CD4⁺) phenotype in B cell chronic lymphocytic leukemia. *Blood* **65**, 149.
9. Inoshita T. & Whiteside T. L. (1981) Imbalance of T-cell subpopulations does not result in defective helper function in chronic lymphocytic leukemia. *Cancer* **48**, 1754.
10. Chiokazzi N., Fu S. M., Montazeki G., Hunket H. G., Rai K & Gee T. (1979) T-cell helper defect in patients with chronic lymphocytic leukemia. *J. Immun.* **122**, 1087.
11. Foa R., Lauria F., Catovsky D. & Galton D. A. G. (1982) Reduced T-colony forming capacity in B-chronic lymphocytic leukemia—11. Correlation with clinical stage and findings in B-prolymphocytic leukemia. *Leukemia Res.* **6**, 329.
12. Lauria F., Foa R., Mantovani V., Fiero M. T., Catovsky D & Tura S. (1983) T-cell functional abnormality in B-chronic lymphocytic leukemia: evidence of a defect of the T-helper subset. *Br. J. Haemat.* **54**, 277.
13. Bloem A. C., Clevers J. C., Bast E. J. E. G. & Ballieux R. E. (1986) T cells in B-cell chronic lymphocytic leukemia—1. Decreased frequency of T lymphocytes secreting suppressor factor. *Clin. exp. Immun.* **63**, 188.
14. Kay N. E. (1981) Abnormal T-cell subpopulation function in CLL: excessive suppressor (CD4) and deficient helper (CD4) activity with respect to B-cell proliferation. *Blood* **57**, 418.
15. Sieber G. & Herrmann F. (1983) Increased suppressor- and normal helper T-cell activity in patients with B-cell chronic lymphocytic leukemia (B-cell). *Br. J. Haemat.* **55**, 561.
16. Terstappen L. W. M. M., de Grooth B. G., ten Napel C. H. H., van Berkel W. & Greve J. (1986) Discrimination of human cytotoxic lymphocytes from regulatory and B-lymphocytes by orthogonal light scattering. *J. Immun. Methods* **95**, 211.
17. Terstappen L. W. M. M., de Grooth B. G., Nolten G. M. J., ten Napel C. H. H., van Berkel W. & Greve J. (1986) Physical discrimination between human T-lymphocyte subpopulations by means of light scattering, revealing two populations of CD8-positive cells. *Cytometry* **7**, 178.
18. Rai K. R., Sawitsky A., Cronkite E. P., Chanana A. D., Levy R. N. & Pasternack B. S. (1975) Clinical staging of chronic lymphocytic leukemia. *Blood* **43**, 219.
19. Nicholson J. K. A., Jones D. M., Cross G. D. & McDougal J. S. (1984) Comparison of T- and B-cell analyses on fresh and aged blood. *J. Immun. Methods* **73**, 29.
20. Knapp M. S. & Pawnall R. (1984) Lymphocyte arithmetics: is this important? *Br. Med. J.* **289**, 1328.
21. Dickinson A. M., George S. & Proctor S. J. (1983) T-cell subpopulation in CLL: methods of T-cell enrichment artificially alter proportions of OKT4 and OKT8 positive cells. *Clin. exp. Immun.* **54**, 525.
22. Deegan M. J., Hayashi H. & Sawdyk M. (1983) Sheep erythrocyte rosette formation by B-chronic lymphocytic leukemia cells as a consequence of monoclonal surface immunoglobulin with 'Forsman-like' antibody activity. *Am. J. clin. Path.* **80**, 69.
23. Worman C. P. & Cawley J. C. (1982) B-CLL cells express true endogenous E receptor after culture with T cells and mitogens. *Br. J. Haemat.* **52**, 205.
24. Foa R., Launia F., Lusso P., Biubellino M. C., Ficuro M. T., Fernando M. L., Raspadori D. & Malera L. (1984) Discrepancy between phenotypic and functional features of natural killer T-lymphocytes in B-cell chronic lymphocytic leukemia. *Br. J. Haemat.* **58**, 509.
25. Foa R., Fierro M. T., Lusso P., Raspadori D., Fernando M. L., Matera L., Malavasi F. & Launia F. (1986) Reduced natural killer T-cells in B-cell chronic lymphocytic leukemia identified by three monoclonal antibodies: leu-11, A10, AD8. *Br. J. Haemat.* **62**, 151.
26. Platsoucas C. D., Fernando G., Gupta S. L., Kempin S., Clarkson B., Good R. A. & Gubla S. (1980) Defective spontaneous and antibody-dependent cytotoxicity mediated by E-rosette-positive and E-rosette-negative cells in untreated patients with chronic lymphocytic leukemia: augmentation by *in-vitro* treatment with interferon. *J. Immun.* **125**, 216.