T cells in B cell chronic lymphocytic leukaemia. I. Decreased frequency of T lymphocytes secreting suppressor factor

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SUMMARY

A reverse T cell plaque assay was employed to study the ability of purified T cells isolated from the blood of seven patients with B cell chronic lymphocytic leukaemia (B-CLL) to secrete antigen-specific helper or suppressor factors (ThF and TsF respectively) after activation in vitro. It was found that in spite of the phenotypical presence of CD8⁺ cells, the frequency of TsF-secreting cells was strongly decreased as compared to normal values. T cells secreting ThF could be generated in all B-CLL patients tested in about normal frequencies. These results may indicate a tumour induced change in the distribution of cellular subsets within the CD8⁺ cell compartment.

Keywords  B cell chronic lymphocytic leukaemia  T cell  suppressor factor secretion

INTRODUCTION

The functional properties of the T cells present in the blood of patients with chronic lymphocytic leukaemia (CLL) of the B cell type have been subject of many studies. In most of these investigations the regulatory capacities of T cells have been assayed using mitogen driven culture systems. Conflicting data have been reported which include increased (Kay, 1981; Keller et al., 1981; Herrmann et al., 1983), normal (Lauria et al., 1983; Fernandez, MacSween & Langley, 1981) or impaired (Han et al., 1981) T suppressor cell activity and decreased (Kay, 1981; Lauria et al., 1983; Han et al., 1981; Chiorazzi et al., 1979) T helper cell function.

Recently a reverse T cell plaque assay has been developed which allows for an analysis of regulatory T cell activity at a single cell level (Primi, Lewis & Goodman, 1979; Cohen, Litvin & Winfield, 1981). This method has been introduced in our laboratory for the detection of T cells secreting antigen-specific helper (ThF) or suppressor factors (TsF) using rabbit antibodies directed to the constant part of the ThF- and TsF-molecules respectively (Clevers et al., submitted). It was found that close contact between T cells and xenogeneic erythrocytes leads to activation of T helper (Th) and T suppressor (Ts) cells resulting in the formation of ThF-plaque forming cells (ThF-PFC) as well as TsF-PFC. The present study deals with the analysis of ThF- and TsF-PFC formation after activation of the T lymphocytes of seven B-CLL patients with sheep- or ox-erythrocytes (SE, OxE). The results obtained indicate that ThF-PFC can be generated in all patients tested, the frequency of TsF-PFC however was found to be strongly decreased as compared to normal values.

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Lymphocyte isolation and characterization. Lymphocytes were isolated from heparinized blood from patients with CLL by Ficoll-Isopaque centrifugation (d = 1.077 g/ml). Membrane bound immunoglobulins (mlg) on peripheral blood lymphocytes (PBL) were determined using affinity purified FITC- and TRITC-labelled antibodies as described before (Bloem et al., 1982; 1984). The T cells were recognized by the presence of cluster of differentiation (CD) antigens, CD3 (pan T), four (helper/inducer) and eight (cytotoxic/suppressor) antigens were visualized using anti-leu4, anti-leu3a and anti-leu2 monoclonal antibodies (MoAb's) respectively (Becton & Dickinson, Mtn. View, USA). These antibodies were directly conjugated either to FITC (anti-leu4 and anti-leu3) or to biotin (anti-leu2). Visualization of the CD3+ T cells was performed by a second incubation step using TRITC-labelled avidine (1 µg/ml, Becton & Dickinson, Mtn. View, USA).

Isolation of T cells by nylon wool filtration. This was performed using the method described by Julius, Sompson & Herzenberg (1973). Briefly, leukopak-wool (Fenwal lab. IL, USA) was washed in distilled water, packed in 10 ml syringes and autoclaved. A syringe was preincubated with MEM plus 10% human AB serum (all AB serum was heat-inactivated and extensively absorbed with SE) for 30 min at 37 °C. A maximum of 50 x 10^6 PBL were layered on the column and incubated for 30 min at 37 °C. The column was slowly eluted with MEM/10% AB serum (37 °C). The cells obtained by this method contained more than 70% CD3+ T cells as determined by immunofluorescence and will be referred to as Tn.

Tn cell activation and culture. Earlier studies have shown that Tn cells can be activated by interaction with OXE or SE (Clevets et al., submitted). This was achieved in the present investigation by centrifugation of the Tn cells with a 50-fold excess of erythrocytes (10 min, 200 g) in polystyrene tubes (12 x 75 mm, Falcon, Oxnard, USA). The pellet was incubated for 30 min at 0 °C, after which the erythrocytes were removed by NH4Cl-induced lysis. The cells were washed twice and cultured for 4 days in tissue culture tubes (12 x 75 mm, Falcon, Oxnard, USA) in a concentration of 0.5 x 10^6 Tn/ml and a final volume of 3 ml RPMI-1640 supplemented with antibiotics, glutamin (2mm) and 10% AB serum.

Preparation of antisera directed against ThF and TsF. The production of the antisera recognizing antigen-specific T cell factors as well as the criteria of the specificity of these antisera has been described by Kontiainen & Feldmann (1979). In short, ThF- and TsF-containing supernatants were produced by culturing human T cells with the appropriate concentrations of the antigen ovalbumin (OA, 3 µg/ml in the presence of 5% adherent cells or 100 µg/ml without adherent cells respectively). The factors were purified by affinity chromatography on OA-sepharose 4B columns. The acid eluate contained OA-specific helper or suppressor activity respectively. Eluates were stored at -80 C. Rabbits were immunized by subcutaneous injection of 1 ml of eluate in Freund’s Complete Adjuvant (1:1) into four sites in the flanks every week for 6 months. Sera were tested for the capacity to neutralize freshly prepared ThF or TsF, specific for OA or sheep erythrocytes, and were shown to recognize common epitopes on the helper or suppressor factor respectively (Heijner et al., 1982b). Five batches of each anti-ThF and anti-TsF antibodies were prepared which were all effective in neutralizing the antigen-specific factors.

All batches of anti-ThF and anti-TsF antibodies were found to be effective as developing antiserum in the reverse T cell plaque assay. Optimal end-concentrations varied with the antisera; however all antisera could be used at 1:30 and 1:100 dilution. Pre-immune serum did not develop reverse T cell plaques.

Reverse T cell plaque assay. The procedure as described by Clevets et al. (submitted) was applied. In short: to 1 ml of packed Oxe 10 ml of a freshly prepared CrCl3 solution (1 mg/ml) was added plus 0.5 ml of a protein A (prot A) solution (1mg/ml in 0.9% saline) (Pharmacia, Uppsala, Sweden). These coated Oxe were used to prepare a monolayer in a 96 wells flat bottom plate (Falcon, Oxnard, USA), pretreated with poly-l-lysine (m = 60 kD, 100 µg/ml in H2O, Sigma Chemical Co., St Louis, Missouri, USA). The anti-ThF or anti-TsF antibodies (diluted in MEM plus 1% Bovine serum albumine (BSA) were added along with 20 µl of the cell suspension to be tested (2.5 x 10^6 cells/ml in MEM plus 1% BSA). The plate was incubated for 60 min at 37°C. after which the complement was
added (20 μl per well). Pooled guinea pig serum (stored at −80°C, absorbed with prot A-OxE) was used as a source of complement. After incubation for an additional 2 h at 37°C, plaques were counted with the use of an inverted microscope. Plaque forming cells were designated ThF-PFC or TsF-PFC, dependent on the specificity of the developing antiserum used. All assays were performed in duplicate.

From each combination of cells three serial dilutions were assayed. Using this assay system Clevers et al. (submitted) showed that the production of ThF and TsF was localized in respectively the CD4⁺- and CD8⁺-cell subpopulation.

Activation of T cell depleted PBL of the CLL patients, with either OxE or SE did not result in the generation of TsF- or ThF-PFC (data not shown).

RESULTS

PBL of seven patients with B-CLL were isolated and the percentages of T and B cells determined (Table 1). Enriched T cell fractions were obtained by filtration of the PBL over nylonwool. The percentage of T cells present in these fractions varied between 21 and 79%. The CD4/CD8 ratio's differed among the individual patients. This was not due to the filtration procedure as no change in the CD4/CD8 ratio was observed after nylonwool filtration as compared to the ratio's determined in the PBL (Table 1). The ability of normal and CLL T cells to generate helper- and suppressor-factors (ThF and TsF, respectively) after OxE or SE contact was studied using a reverse haemolytic plaque assay. The results are shown in Table 2. In the great majority of the patients studied we could demonstrate, after activation of the Tn cells with erythrocytes, the presence of ThF-PFC (SE: six out of seven patients; OxE: five out of seven patients) and the absence of (below the detection limit) or decreased numbers of TsF-PFC (SE: all patients except Ho; OxE: all patients). In most patients a significant number of cells spontaneously secreting ThF could be detected.

For five CLL patients the calculated frequencies of T cells within the CD4⁺- or CD8⁺-cell compartment producing respectively ThF or TsF after activation with the erythrocytes are given in Table 3. The frequency of cells within the CD4⁺ T cell compartments producing ThF after SE activation was found to be normal in four patients. After OxE activation between 1:30 and 1:5700 CD4⁺ T cells secreted ThF. The occurrence of CLL T cells within the CD8⁺ compartment producing TsF after erythrocyte activation was reduced in all patients as compared to normal values, with the exception of pat. Ho.

**Table 1.** Characterization of PBL from seven patients with B cell chronic lymphocytic leukaemia

<table>
<thead>
<tr>
<th>Patient</th>
<th>Lymphocyte count (×10⁶ per cm³)</th>
<th>B cells (%)*</th>
<th>T cells (%)*†</th>
<th>PBLCD4⁺CD8⁺</th>
<th>TnCD4⁺CD8⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col</td>
<td>6-7</td>
<td>73 (μόχ)</td>
<td>10</td>
<td>0-9</td>
<td>1-0</td>
</tr>
<tr>
<td>Cor</td>
<td>8-0</td>
<td>78 (μόλ)</td>
<td>21</td>
<td>2-2</td>
<td>2-6</td>
</tr>
<tr>
<td>Li</td>
<td>29-6</td>
<td>83 (μόλ)</td>
<td>9</td>
<td>1-4</td>
<td>1-5</td>
</tr>
<tr>
<td>Dr</td>
<td>42-0</td>
<td>90 (μόλ)</td>
<td>4</td>
<td>0-9</td>
<td>0-8</td>
</tr>
<tr>
<td>vD</td>
<td>100-0</td>
<td>90 (μόλ)</td>
<td>3</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>vZ</td>
<td>11-0</td>
<td>95 (μόλ)</td>
<td>4</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Ho</td>
<td>11-7</td>
<td>70 (μόλ)</td>
<td>14</td>
<td>2-4</td>
<td>2-5</td>
</tr>
</tbody>
</table>

* Characterization and percentage of monoclonal B cells present in PBL, as determined by immunofluorescence.
† Percentage of CD3⁺ T cells present in PBL, as determined by immunofluorescence.
‡ The ratio of CD4⁺ T cells and CD8⁺ T cells present in PBL.
* Idem for nylonwool purified T cells.
nd Not done.
Table 2. Number of ThF- and TsF-PFC present in $1 \times 10^4$ normal or B CLL Tn cells, induced after activation with erythrocytes

<table>
<thead>
<tr>
<th>OxE*</th>
<th>SF*</th>
<th>Control†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ThF-PFC</td>
<td>TsF-PFC</td>
</tr>
<tr>
<td>Col</td>
<td>3,200</td>
<td>80</td>
</tr>
<tr>
<td>Cor</td>
<td>130</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>Li</td>
<td>390</td>
<td>70</td>
</tr>
<tr>
<td>Dr</td>
<td>13,650</td>
<td>100</td>
</tr>
<tr>
<td>vD</td>
<td>1,860</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>vZ</td>
<td>14,200</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>Ho</td>
<td>7,030</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>ND‡</td>
<td></td>
<td>(n = 5)</td>
</tr>
</tbody>
</table>

* Tn cells are activated with OxE or SE as described in material and methods. After 4 days of culture the cells were assessed for helper- and suppressor-factor production. Number of PFC (ThF-PFC and TsF-PFC, respectively) are expressed per million Tn cells.
† Tn cells were subjected to the same procedural actions, without being exposed to the erythrocytes.
‡ Tn cells of normal donors. The number of PFC is expressed as the mean ± s.d.

Table 3. Frequencies of ThF- and TsF-PFC within the CD4⁻- and CD8⁻-T cell compartments of B CLL patients and normal individuals, after activation with erythrocytes

<table>
<thead>
<tr>
<th>OxE</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ThF-PFC*</td>
<td>TsF-PFC†</td>
<td>ThF-PFC*</td>
<td>TsF-PFC†</td>
</tr>
<tr>
<td>Col</td>
<td>1:170</td>
<td>1:6,000</td>
<td>1:400</td>
<td>1:11,900</td>
</tr>
<tr>
<td>Cor</td>
<td>1:5,700</td>
<td>&lt; 1:20,000</td>
<td>1:530</td>
<td>&lt; 1:20,000</td>
</tr>
<tr>
<td>Li</td>
<td>1:1,700</td>
<td>1:6,000</td>
<td>&lt; 1:20,000</td>
<td>&lt; 1:20,000</td>
</tr>
<tr>
<td>Dr</td>
<td>1:30</td>
<td>1:5,800</td>
<td>1:580</td>
<td>&lt; 1:20,000</td>
</tr>
<tr>
<td>Ho</td>
<td>1:200</td>
<td>&lt; 1:20,000</td>
<td>1:370</td>
<td>1:640</td>
</tr>
<tr>
<td>ND‡</td>
<td>1:240-1:350</td>
<td>1:300-1:380</td>
<td>1:430-1:690</td>
<td>1:590-1:750 (n = 5)</td>
</tr>
</tbody>
</table>

* calculated frequencies of CD4⁻ T cells producing ThF (ThF-PFC) after activation with OxE or SE.
† calculated frequencies of CD8⁻ T cells producing TsF (TsF-PFC) after activation with OxE or SE.
‡ ND normal donor. The values are expressed as a minimum and a maximum frequency, as a result of interdonor differences.

DISCUSSION

Chronic lymphocytic leukaemia is usually a malignancy of B cell origin, characterized by a monoclonal expansion of B cells. In spite of the low percentage of T cells in the PBL of the majority of patients, the absolute number of T lymphocytes per cm² exceeds that of normal individuals.
(Catoovsky et al., 1974). The data presented in this paper show that in most CLL patients studied, T cells able to develop into TsF-PFC activation with O xoE or SE, are present in a strongly reduced frequency (Table 3).

Uytdede Haag et al. (1981) have reported that the CD8+ T cell subset of normal human PBL includes Ts effector cells that are able to secrete antigen-specific TsF after proper stimulation. In addition it has been shown by Clevers et al. (submitted) that T cells which develop into TsF-PFC are of the CD8+ phenotype.

In the patients the absolute increase in the number of circulating T lymphocytes includes both the CD4+ and CD8+ cells (Table 1). This observation leads to the question whether the low numbers of TsF-PFC in these patients is due to a functional defect or is caused by a virtual absence of Ts cells within the CD8+ T cell subpopulation. Since single reagents (e.g. monoclonal antibodies) which can define Ts cells within the CD8+ T cell subset are not available, no definitive conclusion regarding the cause of the impaired formation of TsF-PFC can be drawn.

In a separate study it was observed that also antigen-specific Ts precursor cells, as detected in a functional assay using ovalbumin as an antigen (Heijnen, Pot & Ballieux, 1982a), are strongly reduced or even absent in PBL of CLL-patients (Bloem et al., 1985). This may indicate that the reduced and sometimes virtually absent Ts effector function, reported in the present paper, actually reflects a lack of (functional) precursor Ts cells.

The lack of Ts-activity in conjunction with an increase in the absolute number of CD8+ cells in the blood of CLL patients may indicate that distributional changes have occurred within the CD8+ cellular compartment. This shift may involve non-antigen specific cytotoxic cells (cells active in the antibody dependent cell-mediated cytotoxicity and natural killer cells), which reportedly may express CD8 antigens (Ortaido et al., 1981; Abo, Cooper & Balch, 1982; Larrier et al., 1983; Wählín & Perlmann, 1983).

Another explanation may relate to the excessive presence of single immunoglobulin variable (V)-region associated structures on the leukemic B cells. V-region sequences can elicit both cytotoxic T cell- (Sherman, 1982; Snodgrass, Bosma & Wilson, 1981) and V-region restricted Ts cell-responses (Zubler, Benacerraf & Germain, 1980). In the first case this could result in a shift towards cytotoxic cells within the CD8+ cell compartment, in the second case this could cause a shift towards clonally restricted suppressor T cells. Both assumptions may explain the virtual absence of antigen-specific Ts cells after activation with an arbitrary exogenous antigen.

Clearly more functional studies on the antigen-specific level are needed to determine the exact immunoregulatory and effector functions of T cell subsets from patients with B CLL.

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REFERENCES


