Phorbol Ester Induces Abnormal Chronic Lymphocytic Leukemia Cells to Express Features of Hairy Cell Leukemia


We have investigated the relationship between chronic lymphocytic leukemia (CLL), hairy cell leukemia (HCL), and different normal B cell subsets: Mrbc*, T1* and slgM* tonsil cells; germinal center; mantle zone; and peripheral blood B lymphocytes. Both malignant and normal cells were incubated in vitro with the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) for 72 hours and the morphology, cytochemical profile, and surface markers were evaluated. The results show that CLL cells TPA-induced become indistinguishable from HCL by four independent criteria: the morphology; the cytoplasmic tartrate-resistant acid phosphatase (TRAP) enzyme activity; the membrane positivity with anti-Leu M5 (SHCL3); and anti-Tac monoclonal antibodies which, in the uninduced state, react only with HCL. The features of TRAP and Tac positivity are also expressed (though in variable degree) by different normal B cell populations activated with TPA or pokeweed mitogen (PWM). It is concluded that HCL might represent an aberrantly activated variant of CLL (or of a CLL-related disorder).

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MATERIALS AND METHODS

Preparation of cells. Peripheral blood lymphocytes (PBL) were obtained from 24 patients. Sixteen patients had B-CLL (10 men and 6 women; aged 49 to 70 years); 7 patients were in stage 0-1 (Rai, ref. 13); 4 were in stage II; 4 were in stage III; and 1 patient was in stage IV. None of the patients received chemotherapy during the four months preceding the analysis. The B lymphocytes in all patients expressed monoclonal Ig (k or \( \lambda \)') with a low intensity and formed rosettes with Mrbc (Table 1).

Four patients with HCL were seen (all men; aged 52 to 68 years). The diagnosis was based on cell morphology, the presence of 50% to 75% TRAP* cells in the peripheral blood and in the bone marrow (BM) as shown in trephine biopsies.

One patient (a 63-year-old man) had B cell prolymphocytic leukemia (PLL) with strongly slgM* circulating lymphocytes that did not form Mrbc rosettes (Table 2).

Finally, three patients were diagnosed as having mantle zone lymphoma as defined by Palutke et al.14 The patients were in leukemic phase with strongly slgM* and Mrbc* circulating monoclonal B lymphocytes (Table 2).

Normal PBL samples were obtained from five adult volunteers.
The cells were gently resuspended, transferred onto FH, C5 cells. Among B cell malignancies, SHCL3 was negative with non-

\( 5 \times \) incubating tonsil lymphocytes receptor. Waldmann (National Institutes of Health, Bethesda) identified IL-2 cells (negative).

**differential expression on T cells and B cells in the mantle zone of washed and used as 107/mL concentration. fluorescence (IF).**

and Cappel Labs (Cochranville, Pa) and used in direct immuno-

lymph nodes (positive) as opposed to germinal center (GC) blast
to HLA-DR la-like and to T cell-specific T3-like antigen (Leu4) nate (FITC) Becton Dickinson (Mountain View, Calif). The McAbs (IL, isotype

phosphate-buffered saline (PBS) and resuspended in RPMI medium

were obtained during standard staging procedures from patients (three men and two women aged 27 to 36 years). Four BM samples were

Mononuclear cells were separated from blood or BM on Ficoll-

Hypaque (FH). The cells at the interphase were washed with phosphate-buffered saline (PBS) and resuspended in RPMI medium supplemented with 10% fetal calf serum (FCS).

Tonsil samples were obtained from seven children undergoing tonsillectomy after antibiotic treatment. The cell suspensions were washed and used as 10 ml/mL concentration.

**Antibodies.** Rabbit (R) and goat (G) antisera to human Ig isotype (a, A, s, A) labeled with tetrachloro-6-diamino-3-isoctoy-

nate (TRITC) were purchased from Dakopatt (Glostrup, Denmark) and Cappel Labs (Cochranville, Pa) and used in direct immuno-

fluorescence (IF).

McAbs were investigated in indirect IF assays with G-anti-mouse (M)-IgG or rat-anti-M-1gM conjugated with fluorescein isothiocya-

nate (FITC) Becton Dickinson (Mountain View, Calif). The McAbs to HLA-DR Ia-like and to T cell-specific T3-like antigen (Leu4) were from Becton Dickinson. The McAb to T1-like T cell associated antigen (p67) was RFT1.11 RFA-2 Ab detected an antigen with

labeled with tetraethylrhodamine-isothiocya-

Table 1. TPA (1.6 x 10^-8 mol/L)-Induced Changes in B-CLL, Mrbc' Normal B Cells and HCL Populations

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>72 h Without TPA</th>
<th>72 h With TPA</th>
<th>0 h Without TPA</th>
<th>72 h With TPA</th>
<th>0 h Without TPA</th>
<th>72 h With TPA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphicology</strong></td>
<td><strong>Small</strong></td>
<td><strong>Small</strong></td>
<td><strong>Hairy</strong></td>
<td><strong>Small</strong></td>
<td><strong>Small</strong></td>
<td><strong>Immuno</strong></td>
</tr>
<tr>
<td><strong>B-CLL-associated features†</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mrbc⁺</td>
<td>16/16 (60)</td>
<td>16/16 (43)</td>
<td>0/16</td>
<td>4/4 (79)</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>RFT1⁺</td>
<td>16/16 (89)</td>
<td>16/16 (88)</td>
<td>16/16 (87)</td>
<td>4/4 (71)</td>
<td>4/4 (70)</td>
<td>4/4 (45)</td>
</tr>
<tr>
<td>Sig⁺</td>
<td>16/16 (80)</td>
<td>16/16 (80)</td>
<td>0/16</td>
<td>4/4 (84)</td>
<td>4/4 (80)</td>
<td>0/4</td>
</tr>
<tr>
<td>Cytopl. Ig⁺</td>
<td>0/16</td>
<td>0/16</td>
<td>12/16 (50)</td>
<td>0/4</td>
<td>0/4</td>
<td>4/4 (85)</td>
</tr>
<tr>
<td><strong>HCL’-associated features†</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHCL3⁺</td>
<td>0/16</td>
<td>0/16</td>
<td>13/16 (75)</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Tac⁺</td>
<td>1/16 (80)</td>
<td>1/16 (80)</td>
<td>9/16 (80)</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>TRAP⁺</td>
<td>0/16</td>
<td>0/16</td>
<td>16/16 (73)</td>
<td>0/4</td>
<td>0/4</td>
<td>2/4 (20)</td>
</tr>
<tr>
<td><strong>Mitotic index (%)</strong></td>
<td>0-0.1 NT</td>
<td>0-1 0.5-1 NT</td>
<td>2.5 ± 1</td>
<td>0.1-1 NT</td>
<td>0.5-2</td>
<td></td>
</tr>
</tbody>
</table>

TPA, 12-0-tetradecanoyl-phorbol-13-acetate; Mrbc, mouse red blood cells; ND, not determined on adherent cells which nevertheless failed to bind

layered Mrbc; NT, not tested. SHCL3, anti-Leu MS antibody; TRAP, tartrate resistant acid phosphatase.

The threshold value to define positivity was 20% of reacting cells.

* Numbers of samples studied in each group.

† Number of samples positive/number of samples studied (numbers in parentheses, median values for the positive results).
Table 2. TPA (1.6 × 10⁻⁸ mol/L)-Induced Changes in Strongly Ig⁺ B Cell Malignancies and Normal B Cells of MZ and Germinal Center

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>PLL and MZ Lymphoma† incubated for</th>
<th>Normal B Cells of MZ*† incubated for</th>
<th>Normal Blasts of Germinal Center†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h (n = 4)</td>
<td>72 h (n = 4)</td>
<td>72 h (n = 4)</td>
</tr>
<tr>
<td></td>
<td>Without TPA</td>
<td>With TPA</td>
<td>Without TPA</td>
</tr>
<tr>
<td></td>
<td>(n = 4)</td>
<td>(n = 4)</td>
<td>(n = 4)</td>
</tr>
<tr>
<td>Morphology</td>
<td></td>
<td>72 h (n = 4)</td>
<td>72 h (n = 4)</td>
</tr>
<tr>
<td></td>
<td>with nucleus</td>
<td>with no membrane changes</td>
<td>Blasts</td>
</tr>
<tr>
<td>M1bc⁺</td>
<td>5.66 65.40†</td>
<td>5.45 46.18†</td>
<td>&lt;5†</td>
</tr>
<tr>
<td>RFT1⁺</td>
<td>3.8 10.90†</td>
<td>2.5 11.90†</td>
<td>&lt;5†</td>
</tr>
<tr>
<td>Ig⁺</td>
<td>&gt;5†</td>
<td>&gt;90†</td>
<td>&lt;5†</td>
</tr>
<tr>
<td>SHCL3⁺</td>
<td>&lt;5†</td>
<td>&lt;5†</td>
<td>&lt;1†</td>
</tr>
<tr>
<td>anti-Tac⁺</td>
<td>&lt;5†</td>
<td>&lt;5†</td>
<td>&lt;1†</td>
</tr>
<tr>
<td>TRAP⁺</td>
<td>&lt;10†</td>
<td>20.5 9.15†</td>
<td>&lt;1†</td>
</tr>
<tr>
<td></td>
<td>Percentage of cells reacting with each marker</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TPA, 12-o-tetradecanoyl-phorbol-13-acetate; MZ, mantle zone; PLL, prolymphocytic leukemia; W, weak; SHCL3⁺, anti-Leu M5 antibody; TRAP, tartrate-resistant acid phosphatase.

For details, see Table 1.

*The separated RFA-2⁺ cells included B cells of the MZ and T cells. In the culture B cells were identified by Ig-positivity. Values shown are percentages within the Ig⁺ population.

†Percentage of cells reacting with each marker.
adhesiveness is so pronounced that it is virtually impossible to harvest the induced cells without serious damage. Therefore, HCL cells were cultured inside the vessels directly onto glass coverslips using the same conditions. In each patient, duplicate cultures were set up as controls and as TPA-stimulated samples. TPA was first dissolved in acetone (1.6 x 10⁻⁷ mol/L) and then in absolute alcohol (1.6 x 10⁻⁸ mol/L). The final TPA concentration in the cultures was 1.6 x 10⁻⁶ mol/L, which in previous experiments proved to be optimal for inducing phenotypic changes without major decrease in viability. The cells were harvested and analyzed after 72 hours for morphology, cytochemistry, and membrane markers.

Pokeweed mitogen (PWM) cultures were also established in flat bottomed 96-microtitre plate (Nunc, Copenhagen); 5 x 10⁴ PBLs (depleted of adherent and Srbc T cells) were cocultured with an equal number of autologous Srbc  (T) lymphocytes in a final volume of 0.2 mL per well in the presence of 0.4 μg PWM (GIBCO, Paisley, Scotland) per well. Cultured cells were grown for seven days. The viability was tested, and cytopsins were made.

RESULTS

Definition of B cell populations. B-CLL cells (16 samples) showed the typical features of Mrbc rosetting (Mrbc⁺), reactivity with RFT1 (detecting Tl antigen), and a weak slg expression (Table 1). The HCL-associated markers, such as TRAP enzyme and reactivity with Abs SHCL3 and anti-Tac were absent, except that one case showed a dot-like anti-Tac positivity on 80% of cells (Table 1).

The individual markers did not discriminate fully between B-CLL and other B cell chronic malignancies. Among patients with the four typical forms of HCL, one patient had 65% RFT1⁺ cells, and all four patients had a few (33% ± 4%) Mrbc⁺ cells. Nevertheless, these HCL patients were strongly positive for SHCL3 (84% ± 7%), Tac antigen (84% ± 4%) and TRAP (60% ± 5%, Table 1). Among the other patients with B cell malignancies with very strong Ig expression (PLL and mantle zone lymphoma), three had 40% to 68% Mrbc⁺ cells. Nevertheless, the clinical and histological features and the overall phenotypic findings together clearly defined the three disease groups.

The normal Mrbc⁺ B cells separated from tonsil showed similar features to the B-CLL cells (Table 1; see also refs. 11 and 18). The circulating B cells (Table 3) and those in the mantle zone (Table 2) appeared to correspond to the features of strongly slg⁺ B cell malignancies and not to those of B-CLL (see also ref. 12).

Induction of HCL-associated features in B-CLL. Sixteen cases of B-CLL were incubated for 72 hours with or without TPA. The viability in both groups was >85% during the incubation period. The mitotic index was low (0% to 1%). In the cultures without TPA, the cells remained small lymphocytes. The percentage of Mrbc⁺ cells decreased from 61% ± 5% at 0 hours to 46% ± 16% at 72 hours, but no other phenotypic changes were seen. In contrast, in the TPA-stimulated cultures, the cells became larger, acquired a clearly visible nucleolus and basophilia in the cytoplasm.

Striking membrane irregularities such as veils, protrusions, and microprocesses also developed (Figs 1 a through d, e), and those irregularly shaped cells were apparent in all cases tested. Individual variations in the proportion of these cells were observed, however: in six cases, the percentage of cells with membrane aberrations was between 30% and 50%, whereas in the remaining ten cases, more than 80% of induced cells had hairy-cell like morphology. The slg disappeared and moderately strong cytoplasmic Ig (μ only without δ, γ, or α) was expressed in 12 of 16 cases. These cells, after induction, did not form Mrbc rosettes although they remained RFT1-positive. The most striking changes, however, were the induction of HCL-associated features. These were the expression of SHCL3 (in 13 cases, with 74% ± 11% positive cells; Fig 2), that of Tac antigen (in nine cases, 64% ± 21% positive cells; Fig 3) and the appearance of TRAP enzyme (in virtually all cases; 60% to 85% positive cells, Fig 1 A through D). All three features (SHCL3⁺, Tac⁺, and TRAP⁺) were strongly expressed by the "induced" B-CLL cells.

In contrast to B-CLL, the TPA-activated PLL and mantle zone lymphoma did not develop the full set of HCL-associated features. The cells became larger; however, no

| Table 3. Phenotype Features of B Cells in the Blood and Bone Marrow |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Phenotype | B Cells in Peripheral Blood incubated for | | | |
| | 0 h (n = 3) | 72 h Without TPA (n = 3) | 72 h With TPA (n = 3) | 7 Days With PWM (n = 3) | B Cells in Bone Marrow (n = 4) |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Morphology | Small lymphocytes | Small lymphocytes | Large cells with basophil cytoplasm (no membrane changes) | B blasts and plasma cells | Small lymphocytes |
| Mrbc⁺ | 18, 27, 25 15, 15, 10 | <5 | <1 | <1 |
| RFT1⁺ | <1 | <1 | <1 | <1 |
| Ig⁺ | 100 | >90 | >90 | 24, 25, 27 | 3, 24 |
| SHCL3⁺ | <1 | <1 | 18, 21, 22 | 2, 10, 12 | 7, 8, 13 |
| anti-Tac⁺ | <1 | <1 | <1 | 5, 7, 4 | <1 |
| TRAP⁺ | <1 | <1 | 18, 20, 23 | 60, 65, 55 | <1 |

B lymphocytes were recognized by their Ig positivity. Percentages within the Ig⁺ population are shown. For details see Tables 1 and 2.

PWM, pokeweed mitogen; TPA, 12-0-tetradecanoyl-phorbol-13-acetate; SHCL3⁺, anti-Leu M5 antibody; TRAP, tartrate-resistant acid phosphatase.
TPA INDUCES CLL TO ACQUIRE HCL FEATURES

Fig 1. Tartrate-resistant acid phosphatase (TRAP) staining of chronic lymphocytic leukemia (CLL) and various normal B cell populations after induction of 12-O-tetradecanoyl-phorbol-13-acetate (TPA). TPA-induced CLL cells acquire membrane perturbations and a strong cytoplasmic TRAP positivity (A through D). Germinal center cells remain TRAP- after TPA activation (E). On the contrary, both mantle zone (F) and peripheral blood (G) B cells become TRAP+. Pokeweed mitogen (PWM)-stimulated peripheral blood B cells become TRAP+ (H). The intensity of TRAP staining in peripheral blood B cells both TPA-activated (G) and PWM-activated (H) is definitely weaker and is represented by few scattered granules.

Membrane alterations were seen, and the SHCL3 positivity also remained low (1% to 8%). Nevertheless, anti-Tac positivity (30% to 80%) and TRAP enzyme accumulation (5% to 20%) was observed (Table 2).

B-CLL and normal Mrbc+ cells respond differently to TPA. It was shown above that after TPA induction, B-CLL cells have exhibited HCL-associated features. As controls, normal Mrbc+ cells were isolated from the human tonsil and incubated with TPA (Table 1). These cells lost Mrbc receptor (<5%; also seen without TPA) and had lower proportions of RFT1+ cells (48 ± 5%). The loss of slg and acquisition of cytoplasmic μ (but not δ, γ, or α) were also seen. Nevertheless, the proportions of SHCL3+, anti-Tac+, and TRAP+ cells in these normal cell cultures remained much lower (6.8% ± 2%, <1% and 18% ± 7%, respectively) than in the corresponding TPA-activated B-CLL cultures shown above. Furthermore, TPA-induced normal B cells grew in size, but no membrane perturbations were seen.

Other normal B cell populations were also tested. Strongly Ig+ B cells of tonsil (Table 2) and circulating B cells in peripheral blood (Table 3) were incubated with TPA for 72 hours. Again, no membrane perturbations developed. Few B cells had SHCL3 positivity (20% to 30% and 15% to 25%, respectively). The TRAP enzyme activity was restricted to only a few granules in 15% to 25% of blood B cells (Fig 1g) and in 60% of tonsil B cells (Fig 1f). Only occasional Tac+ B cells were seen in all of these cultures (<1%; Tables 2 and 3), but a few anti-Tac+ B blasts (5.3% ± 1.5%) were observed in cultures stimulated for PWM for seven days (Table 3, Fig 3). Several cells in these PWM cultures had a granular pattern of TRAP positivity (Fig 1h).

Finally, isolated GC blasts (RFA-2; see methods) cul-
SHCL3, cultured for 72 hours in the presence of TPA, remained 85% viable, while findings are seen with normal B cells. In cultures without TPA, the hairy cell morphology appears to be the TI approach is that the normal equivalent cells of B-CLL stimulate B cell leukemias. The rationale for this variability of cells of the lymphohemopoietic lineage and also cell subsets, both malignant and normal, during culture in vitro can show membrane ruffles. However, TPA-induced B-CLL cells show hair-like projections when observed both with phase microscopy and with electron microscopy. We have also recently demonstrated that these membrane perturbations are caused by an anomalous rearrangement of the cytoskeleton that is unique to B-CLL and HCL. Experiments with microcinematography are in progress in order to investigate whether the villous processes shown by B-CLL cells that are TPA induced undergo undulation without locomotion. Our findings do not, however, exclude the possibility that other antisera may find differences between TPA-induced B-CLL cells and HCL. It is already known that B-CLL and HCL may differ in isotype expression. B-CLL cells usually display sIgM and sIgD, while sIgG can only be detected by sensitive rosetting methods. In contrast, HCL cells during TPA induction do not switch their Ig production to IgG. B-CLL and HCL also have a different pattern of organ involvement (reviewed in ref. 25). Nevertheless, the relationship between these two diseases is also shown by intermediate forms with TRAP circulating hairy-looking cells in the peripheral blood and histological features of CLL seen in the spleen and lymph nodes of the same patients. On the other hand, other B cell malignancies such as PLL, mantle zone lymphomas, and follicular lymphomas (unpublished results, F. Caligaris-Cappio, March 1984) do not develop HCL-like features after TPA and do not show intermediate forms with HCL. It may therefore be concluded that B-CLL and HCL cells, although possibly showing minor differences, appear to be relatively closely related disorders.

Second, the response of B-CLL and their normal equivalent Mrbc⁺, T1⁺, sIgM⁺ cells to TPA is different. Membrane perturbations are seen in CLL, but not in their normal counterpart. Furthermore, the degree of activation by TPA in B-CLL cells appears to be more dramatic than that seen in Mrbc⁺, T1⁺, cells of the normal tonsil or in any other B cell subsets studied (Tables 2 and 3). Previous studies had already shown that TPA treatment of CLL increases the intensity of surface Ig and facilitates the expression of “activation” antigens. Furthermore, CLL cells, poor stimulators in mixed lymphocyte cultures, increase their stimulatory capacity after TPA. The markers analyzed in this study are clearly not leukemia-specific. The particularly strong Tac antigen expression on the surface of HCL-induced and TPA-induced B-CLL cells comes closest to being an aberrant phenomenon as it reaches the intensity of Tac antigen expression in T lymphoblasts (Fig 3). However, many B cell lines and occasional B
blasts in PWM cultures of normal peripheral B cells are also strongly Tac\(^+\). A number of groups have recently focused attention on the weaker but definite anti-Tac (IL-2 receptor) expression on activated normal B cells\(^{34,35}\) and suggestive evidence shows that IL-2 can influence B cell differentiation in the absence of T cells.\(^{35}\) Thus, Tac can be regarded as a B cell activation antigen that is particularly strongly expressed on HCL and activated B-CLL. Similarly, only a small proportion of normal B cells are TRAP\(^+\). These are present in the mantle zone of lymph nodes (M. Chilosi, unpublished observations January 1984). Now we show that TPA can induce TRAP activity in a number of B cell types: mantle zone, Mrbc\(^+\), Tl\(^+\), and peripheral blood B lymphocytes. Also, PWM-activated peripheral blood B cells become TRAP\(^+\). There is again one notable exception: blasts of the GC remain TRAP\(^+\), reaffirming the possibility that follicular B cell blasts represent a different lineage or stage of activation (reviewed in ref. 37).

Therefore, HCL-induced and TPA-induced B-CLL appear to represent a particularly strongly activated B cell (see also refs. 12 and 31) with an apparently disturbed membrane that is different from the corresponding normal cell and indistinguishable, using the criteria studied in this paper, from HCL. A corollary of this finding is that some activated normal B cells also show Tac and TRAP positivity. In addition, Posnett et al\(^{11}\) have also shown that the HCL-associated marker HC2 is also expressed by a small number of normal circulating activated B cells. Taken together, these findings indicate that Tl\(^+\) B cell malignancies such as B-CLL as well as HCL appear to represent a closely related disease group with distinct differences from both the follicular lymphomas (GC diseases; ref. 37) and the bone marrow-homing multiple myeloma. Among normal B cell populations, the most likely candidate to be the target cell for the transforming events appears to be the Tl\(^+\), slgM\(^+\) B cell subset and/or its close derivative. The Tl\(^+\), slgM\(^+\) cells were shown to cluster around follicular dendritic cells in primary follicles of fetal lymph nodes\(^{36}\) and are apparently diluted out at the edge of GCs in adult lymph nodes by the emerging secondary follicles.\(^{11}\) The phenotype of Tl\(^+\), slgM\(^+\) cells is reproduced by CLL and centrocytic lymphoma.\(^{11,38}\) Additional gene disturbances may act upon this cell lineage differentiation program and result in an abnormally activated stage and membrane disorders revealing themselves in the features of hairy cells.

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