Analysis of Signal Transduction in B Chronic Lymphocytic Leukemia Cells

By Hans G. Drexler, Malcolm K. Brenner, Elaine Coustan-Smith, Suzanne M. Gignac, and A. Victor Hoffbrand

We report here experiments on the analysis of cellular signal transduction in a series of patients with chronic B cell disorders (B cell chronic lymphocytic leukemia [B-CLL] and prolymphocytic leukemia). We compared the response of the leukemic cells with primary external signals (interleukin 2 [IL-2] or B cell differentiation factors [BCDF or IL-6]) with their response to secondary inducers (the phorbol ester 12-0-tetradecanoylphorbol-13-acetate [TPA] or the calcium ionophore A23187) that circumvent the first part of the signal transduction pathway by directly activating the key enzyme protein kinase C. One BCDF was synthesized by mitogen-activated peripheral blood B lymphocytes; a second BCDF was constitutively produced by the human bladder carcinoma cell line T24. Changes in morphology, Tac (IL-2 receptor) expression, RNA synthesis measured by 3H-uridine uptake, and immunoglobulin production tested by enzyme-linked immunosorbert assay were used as parameters of successful signal transduction.

TPA alone and TPA plus A23187 (synergistically) effectively initiated differentiation in all the leukemia cases. Neither IL-2 nor BCDF (singly or in combinations) caused equivalent responses. On the other hand, IL-2 and BCDF produced a substantial differentiation effect on normal B lymphocytes. Our data suggest that (a) B-CLL cells are able to respond to direct stimulation of the second messenger pathway (through protein kinase C) but not to the physiological stimuli IL-2 or BCDF; (b) the defect in signal transduction appears to be located upstream of protein kinase C (a possible candidate is a G protein); (c) malignant B cells may spontaneously or after treatment with inducers express the IL-2 receptor (Tac antigen) in the absence of a functional differentiating response to IL-2; and (d) signs of proliferation/differentiation in B-CLL samples after incubation with IL-2 or BCDF might be due to contamination of the cell populations with residual normal B cells.

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of PI3, these results show that the signal transduction system downstream of PKC functions in B-CLL cells.

In the present investigation we attempted to answer whether in B-CLL cells the early part of the signal transduction pathway can respond appropriately to external signals such as B cell differentiation factor (BCDF) or interleukin-2 (IL-2). Recently, the designation IL-6 has been proposed for BCDF. As parameters of the end points of the activation events we chose incorporation of $^3$H-uridine to indicate RNA synthesis and production of monoclonal immunoglobulin (Ig).

MATERIALS AND METHODS

Patients and normal controls. Seventeen patients with chronic B cell disorders were selected from a routine hematology clinic. Emphasis was put on choosing patients with WBC counts of at least 30 x 10$^6$/L. Each patient was tested on at least two occasions. Fourteen patients had CLL; the remaining three cases were prolymphocytic leukemia (PLL) n = 2) or CLL in lymphoblastic transformation (CLL/PL, n = 1) as judged morphologically by the number of prolymphocytes present. Diagnosis was established by morphologic, clinical data, and immunologic studies. Clinical data at the time of sampling are presented in Table 1. Surface marker analysis identified all cases as the B cell types of CLL or PLL. Venous blood was also obtained from three healthy adult volunteers.

Cell preparations. Mononuclear cells were isolated from normal or leukemic heparinized peripheral blood by Ficoll-Hyphaque gradient centrifugation (Lymphoprep, Nyegaard, Oslo). Monocytes were removed from all samples by incubation on plastic dishes (Nunc, Roskilde, Denmark) at 37°C for 90 minutes. T cells were depleted by using neuraminidase-treated sheep erythrocyte rosetting (TCS, Berkshire, UK) for 45 minutes at 4°C followed by Ficoll-Hyphaque gradient centrifugation. In three cases of B-CLL and from all normal samples, autologous T cells were recovered by lysing sheep red cells with ammonium chloride solution. These were irradiated with 2,000 rad to a 4$^6$Co source and added to the wells for coculture experiments at a ratio of 4:1 T:B cells.

Table 1. Clinical Data of Patients Studied

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>Diagnosis</th>
<th>WBC (x 10$^9$/L)</th>
<th>Treatment at Time of Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>73/F</td>
<td>B-CLL</td>
<td>63</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>70/F</td>
<td>B-CLL</td>
<td>60</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>83/M</td>
<td>B-PLL</td>
<td>100</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>68/M</td>
<td>B-CLL</td>
<td>72</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>71/F</td>
<td>B-CLL</td>
<td>52</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>80/M</td>
<td>B-CLL</td>
<td>38</td>
<td>None</td>
</tr>
<tr>
<td>7</td>
<td>66/M</td>
<td>B-CLL</td>
<td>166</td>
<td>None</td>
</tr>
<tr>
<td>8</td>
<td>63/F</td>
<td>B-CLL</td>
<td>35</td>
<td>None</td>
</tr>
<tr>
<td>9</td>
<td>84/F</td>
<td>B-CLL</td>
<td>40</td>
<td>None</td>
</tr>
<tr>
<td>10</td>
<td>64/F</td>
<td>B-CLL</td>
<td>95</td>
<td>None</td>
</tr>
<tr>
<td>11</td>
<td>82/M</td>
<td>B-PLL</td>
<td>67</td>
<td>None</td>
</tr>
<tr>
<td>12</td>
<td>67/M</td>
<td>B-CLL</td>
<td>67</td>
<td>None</td>
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<tr>
<td>13</td>
<td>65/F</td>
<td>B-CLL</td>
<td>46</td>
<td>None</td>
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<tr>
<td>14</td>
<td>64/M</td>
<td>B-CLL</td>
<td>83</td>
<td>None</td>
</tr>
<tr>
<td>15</td>
<td>41/M</td>
<td>B-CLL/PL</td>
<td>92</td>
<td>None</td>
</tr>
<tr>
<td>16</td>
<td>38/M</td>
<td>B-CLL</td>
<td>176</td>
<td>CHOP</td>
</tr>
<tr>
<td>17</td>
<td>83/M</td>
<td>B-CLL</td>
<td>90</td>
<td>None</td>
</tr>
</tbody>
</table>

Abbreviations: C, cyclophosphamide; H, hydroxydaunorubicin; O, Oncovin; P, prednisolone.

After depletion of T cells and monocytes, 90% or more of the cells in the leukemic samples were CD20+ (RFB-7) and surface immunoglobulin–positive with less than 1% T cells (CD3+, Leu-4+) and less than 1% monocytes (CD14+, Leu-M3+). In the normal samples the sheep erythrocyte–negative cell populations (nominal B cell populations) contained 40% to 50% CD20+ B cells, about 5% CD3+ T cells, and 1% to 2% CD14+ monocytes.

Routine immunophenotyping. The surface marker profiles of the samples were determined by using the routine leukemia phenotyping program as performed at the Royal Free Hospital (Department of Immunology). The cells were stained with direct or indirect immunofluorescence in suspension according to the microtiter plate methodology and analyzed under an epi immunofluorescence microscope equipped with appropriate filters.

Briefly, the cells were first incubated with specific murine monoclonal antibodies (MoAbs, Table 2) and then stained with fluorescein isothiocyanate (FITC)- or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat antimouse reagents. Surface immunoglobulin expression was tested with directly FITC or TRITC conjugated, isotype-specific, and light chain–specific goat antihuman immunoglobulin antisera. The results of the phenotyping are shown in Table 2.

RF MoAbs (Table 2) and goat antimouse reagents were produced at the Royal Free Hospital (Department of Immunology); goat antihuman immunoglobulin and goat antimouse isotype-specific antisera were obtained from Southern Biotechnology, Birmingham, AL; Leu reagents from Becton Dickinson, Mountain View, CA; OKT 4 from Ortho Pharmaceutical Corp, Raritan, NJ; Tac from Dr T.A. Waldmann; and M-CS-2 and FMC-7 from Dr J. Minowada.

Culture conditions. Enriched B cells from patients with leukemia or normal donors were cultured alone or with autologous purified T cells in RPMI 1640 medium (Flow Laboratories Rickmansworth, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS; Sera Lab, Sussex, UK), L-glutamine (GIBCO, Uxbridge, UK), and penicillin/streptomycin (GIBCO) at 37°C in a humidified incubator with 5% CO2. Microcultures with 4 x 10$^5$ cells/well (2 x 10$^3$/mL) were set up in flat-bottomed 96-well microtiter plates (Nunc). Inducing agents were simultaneously added at day 0 (or in several experiments serially at different intervals) at the following concentrations: 10-4 or 10-5 mol/L TPA (Sigma Chemical Co., Dorset, UK); 0.75 μmol/L A23187 (Sigma Chemical Co., Milan, Italy); 10 mg/L BCDF; and 10% (vol/vol) T24 supernatant. After onset of the culture period no medium was exchanged or added. At 24-hour intervals from days 1 to 6, cells were harvested for analysis of morphology, RNA synthesis and Ig production.

Flow cytometric analysis of IL-2 receptor expression. Day 0 cells and cells cultured in the presence or absence of stimulants for three days were stained with the Tac MoAb (anti–IL-2 receptor antibody, CD25); this was followed by reaction with FITC-conjugated, isotype-specific F(ab')2 reagents as described earlier. Analysis was performed on an EPICS V cell sortor (Coulter Electronics, Hialeah, FL) using an argon laser exciting at 488 nm. Residual dead cells or cell aggregates were gated out according to forward-angle and 90° light scattering. At least 10,000 viable cells were counted; the results were plotted by using the EASY 8 computer program (Coulter).
recultured with modified Iscove's medium. After 72 hours, the supernatants were harvested and filtered. This medium containing BCDF activity was added to the experimental culture cells at a 10% final volume.

**Bladder carcinoma cell line T24 supernatant.** The human bladder carcinoma cell line T24 is known to constitutively secrete a molecule with BCDF-like activity. Recently, the molecular cloning and structural analysis of the complementary DNA (cDNA) encoding human BCDF (IL-6) from a human T cell line (TCL-Na1) that constitutively produced large amounts of BCDF/BSF-2 has been reported. Comparative analysis of T24 cells and TCL-Na1 cells showed that the same mRNA was transcribed in both cell lines. Other authors suggested that the cDNA cloned from TCL-Na1 cells is identical to the gene encoding interferon-β.

Filtered supernatants from T24 cell line cultures were a generous gift from Dr. John Shields (Institute of Child Health, London). T24 supernatant was added to the culture plates at a final concentration of 10% (vol/vol) which had been shown to be optimal in preliminary experiments. We refer to this supernatant as T24 (-BCDF). T24 supernatant was free of IL-1, IL-2, interferon-α, and interferon-γ activity.

**Morphological studies.** The morphology of cells after cultivation (adherence and formation of clusters) was observed in situ on the cells in the original culture wells under an inverted phase-contrast microscope. Aliquots were also taken to prepare cytospins in an inverted phase-contrast microscope. Shandon-Elliot Cytospin, Sewickley, PA). The preparation was stained with standard May-Grunwald-Giemsa and examined by light microscopy.

**Cell size measurements.** Sizes of cells fixed in 8% formalin were measured by using the forward-angle and 90° light scattering parameter of the flow cytometer. Channel numbers on an arbitrary linear scale representing the mean peaks of each curve were compared.

**RNA synthesis determination.** Cells were pulsed with 1 μCi [5,6-3H]uridine (specific activity, 37 Bequerel (MBq)/mCi; American International, Amersham, UK) for one hour. Cells were harvested with a Multimash 2000 (Dynatech Laboratories, Inc., Billinghurst, UK), and incorporation of 3H-uridine into RNA was determined by standard liquid scintillation β counting (LKB 1210 UltraBeta, LKB Instruments, Bromma, Sweden).

### Table 2. Immunophenotypes of Patients Studied (Before T Cell Depletion)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>CD</th>
<th>Reactivity Pattern</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFD2</td>
<td>—</td>
<td>HLA-DR (class II antigen)</td>
<td>83/9, 89, 98, 99, 85, 86, 97, 78, 84, 87, 88, 94, 90, 90, 90, 90, 90, 94</td>
</tr>
<tr>
<td>RFA2</td>
<td>CD10</td>
<td>Common ALL antigen</td>
<td>0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0</td>
</tr>
<tr>
<td>RFB4</td>
<td>CD32</td>
<td>Pan-B</td>
<td>0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0</td>
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<tr>
<td>RFB8</td>
<td>CD21</td>
<td>Pan-B (CD3 receptor)</td>
<td>6, 5, 84, 76, 83, 60, 95, 68, 79, 1, 80, 90, 75, 76, 0, 87, 90</td>
</tr>
<tr>
<td>RFB7</td>
<td>CD20</td>
<td>Pan-B</td>
<td>84, 83, 95, 94, 84, 63, 96, 86, 80, 80, 81, 92, 76, 78, 80, 90, 92</td>
</tr>
<tr>
<td>Smig M/G</td>
<td>—</td>
<td>Surface Ig heavy chain</td>
<td>82, 90, 1, 96, Und, Und, Und, 96, 86, 86, 80, 98, Und, 90, 78, 99, 90, 92</td>
</tr>
<tr>
<td>Smig A/5</td>
<td>—</td>
<td>Surface Ig light chain</td>
<td>99/1, 99/1, 99/1, Und, Und, Und, 99/1, 1/99, Und, 99/1, 99/1, Und, 99/1, 99/1, 99/1, 99/1, 99/1</td>
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<tr>
<td>RFT2</td>
<td>CD7</td>
<td>Pan-T</td>
<td>2, 7, 1, 5, 12, 33, 2, 22, 13, 1, 10, 4, 14, 13, 2, 1, 3</td>
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<tr>
<td>RFT11</td>
<td>CD2</td>
<td>Pan-T (E rosette receptor)</td>
<td>2, 7, 2, 4, 11, 30, 2, 26, 22, 10, 10, 5, 25, 15, 2, 2, 5</td>
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<tr>
<td>RFT1</td>
<td>CD9</td>
<td>Pan-D</td>
<td>92, 91, 2, 96, 99, 93, 96, 95, 95, 93, 96, 89, 93, 93, 93, 93, 93, 93</td>
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<tr>
<td>RFT12</td>
<td>CD6</td>
<td>Pan-T</td>
<td>96, 6, 1, 20, 94, 90, 97, 78, 95, 86, 95, 94, 15, 93, 1, 92, 95</td>
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<tr>
<td>OKT4</td>
<td>T helper</td>
<td>1, 3, 1, 2, 7, 21, 1, 18, 12, 3, 5, 3, 8, 10, 0, 1, 3</td>
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<tr>
<td>RFT8</td>
<td>CD8</td>
<td>T suppressor/cytotoxic</td>
<td>2, 5, 1, 1, 4, 10, 1, 6, 6, 1, 1, 4, 2, 6, 5, 0, 1, 2</td>
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<tr>
<td>FMC7</td>
<td>—</td>
<td>B cell associated</td>
<td>3, 0, 16, 0, 1, 7, 0, 89, 1, 2, 84, 0, 6, 3, 0, 2, 0</td>
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<tr>
<td>Tac</td>
<td>CD25</td>
<td>IL-2 receptor</td>
<td>0, 2, 2, 17, 1, 4, 0, 0, 0, 0, 91, 0, 85, 13, 78, 1, 0</td>
</tr>
</tbody>
</table>

*References for reagents: RF, reagents; FMC7, Tac, 26, 29. Cluster of Differentiation (CD) as defined by the First, Second, and Third International Workshop on Leukocyte Differentiation Antigens (Paris, 1982; Boston, 1984; Oxford, 1986). 30 Positively reactive in immunofluorescence analysis. 31 Highest positive antibody titers for all murine MoAbs. 32 Patients 2 and 12 were monoclonal for IgM heavy chain.

Quantification of Ig production by ELISA. Ninety-six- well Dynatech ELISA plates were coated overnight with rabbit antihuman IgM (Dako Laboratories, Weybridge, UK) or goat antihuman IgG (Sigma). The respective intermediate washing steps were done with three cycles of phosphate-buffered saline containing 0.02% Tween 20. Unoccupied binding sites (to prevent nonspecific binding) were saturated by incubation with a blocking buffer composed of 0.2% bovine serum albumin and 0.2% gelatin dissolved in 0.1% sodium azide solution for two hours at 37°C. Standard human IgM and IgG were prepared by doubling dilutions in blocking buffer. The variable standards dilutions and aliquots of the unknown Ig-containing supernatants and several dilutions thereof were transferred to the test plates and were incubated for two hours. After washing, appropriate dilutions of alkaline phosphatase-conjugated goat antihuman antibodies specific for IgM or IgG (Sigma) were added for two hours. P-nitrophenyl phosphate (Sigma) was used as a substrate. After 15 to 45 minutes, the released p-nitrophenolate was measured at 405 nm on a multispan ELISA reader (Skatron). Absorbance curves for samples and standards were plotted, and Ig concentrations were calculated by using the Rubycrete ELISA program (London). All samples were tested for IgM and IgG. For the determination of monoclonality the production of κ or λ light chains were examined by using goat antihuman κ or λ antibodies conjugated to alkaline phosphatase as a second layer.

**Cell cycle analysis using Ki-67.** Cytospin slide preparations were fixed with cold methanol and stained for 30 minutes with MoAb Ki-67 (Dako); this was followed by 30 minutes of incubation with FITC-conjugated goat antimouse Ig antibody and analyzed by immunofluorescence microscopy. The Ki-67 negative antigen is expressed on all proliferating cells, preferentially during the late G1, S, M, and G2 phases of the cell cycle whereas cells in the G0 phase consistently lack the antigen.

### RESULTS

**Morphological changes induced in B-CLL cells.** On light microscopy, cells exposed to medium, A23187, IL-2, BCDF, or T24 alone became only slightly enlarged but remained for the most part round. TPA induced the following...
ing morphological alterations: increase in cell size; elongation of the clear and occasionally basophilic cytoplasm; and often bizarre, irregular cell forms. Cells treated with the double stimulus of TPA and A23187 increased in size but remained round or oval, the nuclei were eccentrically located, and the cytoplasm became deeply basophilic with a pronounced perinuclear clear zone. Combinations of TPA or TPA plus A23187 with other reagents did not result in different (or increased) morphological changes other than those described for these stimuli.

The vast majority of cells per well (70% to 90%) displayed the described morphological features after incubation with the respective agents. Less than 1% of the cells in any of the cultures treated with different inducers showed mitosis on cytospin slide preparations. In addition, less than 1% were positive for the cell cycle marker Ki-67 in immunofluorescent assays.

Figure 1 depicts a comparison of cell sizes after treatment with different inducers in a representative case. Only small alterations in cell size were induced by A23187, BCDF, T24, or IL-2. Again, combinations of TPA and A23187 led to a substantial increase in cell size.

Analysis of Tac expression in cultured B-CLL cells. Most samples (except those from patients 11, 13, and 15) were negative or only marginally positive after staining with Tac MoAb. After culture for three days with the double stimulus of TPA plus A23187, 45% (±21%) of B cells from the eight CLL patients tested expressed IL-2 receptors as detected by flow cytometric analysis (Fig 2). Medium, TPA, A23187, IL-2, T24, and BCDF alone also induced IL-2 receptor, albeit at low levels (10% to 30%, which might be due to the culture conditions when using 10% FCS).

To our knowledge, the putative BCDF (IL-6) receptor has not yet been isolated. MoAbs detecting the BCDF receptor have therefore not been available for this study.

RNA synthesis caused by induction of B-CLL cells. In all cases, the RNA synthesis (measured by 3H-uridine incorporation) was significantly higher in TPA- or TPA plus A23187–exposed cultures than in wells incubated with medium, A23187, BCDF, T24, or IL-2 alone or with different combinations of these reagents (Fig 3). In all but one sample (patient 16), TPA and A23187 acted synergisti-

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**Fig 1.** Comparison of cell sizes after culture with different inducers. On day 3 of culture, cells were analyzed, and channel numbers on an arbitrary linear scale representing the mean peaks of each curve were related. The percent increase in cell size was calculated as (size of cultured cells/size of uncultured cells - 1) × 100.

**Fig 2.** Tac antigen expression before (day 0) and after (day 3) treatment of B-CLL cells with TPA and A23187. Curve 1, background staining with irrelevant MoAb (MCS-2); curve 2, Tac staining; panels A, patient 7 (from 0.2% positivity at day 0 to 47% at day 3); panels B, patient 4 (from 17% to 56%). Histograms are from flow cytometric analysis.

**Fig 3.** 3H-uridine incorporation (RNA synthesis) in cells from patients with chronic B cell leukemias. The cells were treated with different individual and combined stimuli. Also shown is the peak 3H-uridine uptake occurring at day 2 or 3 (mean from 17 patients).
cally in inducing RNA synthesis. The further addition of other inducers (BCDF, T24, or IL-2) to TPA or TPA plus A23187 did not have significant effects on $^3$H-uridine uptake.

**Ig production in B-CLL cells treated with inducing stimuli.** Normal peripheral blood B lymphocytes from three normal donors responded strongly to BCDF, T24, and IL-2, whereas TPA and TPA plus A23187 were not effective (Fig 4). Coculture with irradiated T cells amplified these results by a factor of 2 to 3 (data not shown).

In parallel with the change in morphology and uptake of $^3$H-uridine, an increase in the amounts of IgM produced was found when using TPA or TPA plus A23187 in B-CLL cells (Fig 5). In contrast, no significant IgM production was seen in the control, A23187-, BCDF-, T24-, or IL-2-treated cultures during the same period. The two patients who produced IgG showed very similar results. Repeated or delayed addition of IL-2 or BCDF or different amounts of IL-2 (from 50 to 250 units, in most cases 250 units were used) had no positive effects.

Although the response was variable, cells from all patients produced increased levels of Ig in the presence of TPA or TPA plus A23187. In all SmIgM-positive cases this was predominantly IgM, with little or no IgG production. For 11 patients the magnitude of Ig production in response to the double stimulus of phorbol ester and calcium ionophore was significantly greater than that produced by the same leukemic B cells treated with TPA alone. It is not known why in the other cases the coculturing with TPA and A23187 was not effective in a synergistic fashion. Two representative examples for this phenomenon are shown in Fig 6. No obvious correlation with clinical, morphological, or immunologic features could be detected.

**Fig 4.** IgM production of normal B cells stimulated with different inducers. B cells were incubated in the presence of irradiated, autologous T cells for six days (shown are the results of the cell cultures from one normal cell population). The amounts of IgM in the wells treated with TPA plus A23187 plus different combinations were below the sensitivity level of the assay (<50 ng/mL).

**Fig 5.** IgM production of B-CLL cells. Cells were exposed to different single and combined inducers for six days (mean from 13 patients; patients 2 and 12 were monoclonal for IgG with similar results, and patients 3 and 15 showed high spontaneous IgM production).

To examine whether the Ig was the product of leukemic (monoclonal) cells or derived from residual normal (polyclonal) B cells, cultures were also tested for monotypic light-chain expression. In each case only one light chain was detected and corresponded to the surface Ig heavy- and light-chain type (in those cases where it was detectable). In contrast, in the normal B cell cultures both IgM and IgG and both \( \kappa \) and \( \lambda \) light chains were produced.

The addition of purified autologous T cells to wells containing B-CLL cells did not result in the production of increased levels of IgM in any of the three patients tested (data not shown). When unfractionated leukemic cell populations (from three patients) were compared with the B-CLL populations from the same patient that were almost completely depleted of T cells (with less than 1% CD3+ cells), comparable amounts of Ig were produced.

**Fig 6.** IgM production of cells from two representative cases with B-CLL. Cells were treated with different inducers for six days. In patient 16 the response to the double stimulus of TPA plus A23187 was not stronger than that to TPA alone, whereas in patient 4 TPA plus A23187 acted synergistically in inducing IgM production. Interestingly, patient 16 did not respond to a previous cycle of CHOP. Other combinations of inducing reagents were also tested, and the results corresponded to those shown in Fig 5.
As observed previously, 36-37 leukemic B cells from some patients have the ability to produce large amounts of Ig spontaneously. In our study these patients (nos. 3 and 15) had B-PLL or B-CLL/PL. The morphological and functional properties of this type of leukemia have suggested that these cells represent a maturation arrest corresponding to that of physiologically activated B lymphocytes.32,38 When using the panel of inducing reagents these cells could be boosted to produce only slightly increased levels of Ig; here also, TPA and TPA plus A23187 were the most efficient stimuli, and IL-2 or BCDF had little effect.

**DISCUSSION**

The maturation arrest seen in B-CLL and related leukemias reflects a defect in the ability to differentiate beyond a certain stage of maturation.38 In the last decade, there have been an increasing number of reports on differentiation induction experiments with B-CLL cells that can be induced to differentiate when provided with the appropriate signal.38,39

In a variety of cellular systems, the effector response to stimuli relies on signal transduction pathways using second messengers to activate effector kinases. We used the approach of induction of differentiation experiments to study the signal transduction mechanisms that appear to be impaired in B-CLL cells.

From data presented here and from previously reported experiments21 we made these main conclusions; (a) as described previously,1 CLL cells can be triggered by TPA to differentiate. (b) Although A23187 itself does not induce differentiation, it enhances synergistically the effect of TPA on the differentiation of CLL cells; the parameters used suggest maturation towards Ig-secreting cells. (c) Neither IL-2 nor BCDF (from peripheral blood B cells or from the T24 bladder carcinoma cell line) had any effect in the differentiation of CLL cells (singly or in combination with TPA and/or A23187) despite the expression of Tac antigen.

Individual variations in the response to TPA and/or TPA plus A23187 were seen in the series of samples tested. We could not establish which parameters influenced the extent of differentiation induced synergistically by phorbol ester and calcium ionophore and why in some cases the combination of these reagents was not more effective than TPA alone. Heterogeneity in the response of CLL cells to phorbol ester alone has already been documented previously.40 These authors suggested that differences in the response of B leukemic cells to TPA might reflect the underlying heterogeneity of the leukemic cells and might be correlated to slightly different stages at which the maturation arrest occurred.

Increased 3H-thymidine uptake and/or elevated Ig production has been observed in B-CLL cultures treated with IL-2 (IL-2 plus TPA, IL-2 plus anti-µ antibody, or IL-2 plus PHA), which has led a number of authors to suggest that B-CLL cells proliferate or differentiate in response to IL-2.41-45 However, it has not been shown unequivocally that the cells responding with differentiation belonged to the leukemic clone and not to residual normal B cells (for example by the demonstration of clonally restricted light-chain production).

The view that IL-2 can efficiently induce proliferation and/or differentiation in B-CLL cells has recently been challenged. Several groups reported that IL-2 is not able to promote the proliferation or differentiation of leukemic B cells.34,46-50 Although IL-2 can induce significant proliferation and differentiation of activated normal B cells,51 moreover, the 3H-thymidine uptake of B-CLL cells cannot necessarily be equated with "proliferation" because no increase in cell number or mitotic index has been reported.

Similarly, different B cell differentiation factors (including T24-BCDF) and other lymphokines (for example, interferons) were described to cause differentiation of B-CLL cells.34,48,52-54 Again, production of monoclonal light chains was not documented in any of the reports. Benjamin et al56 could clearly show that T and B cell–derived lymphokines influence the normal B cell response but not that of leukemic B cells. It is therefore difficult to assess whether groups observing differentiation in the presence of IL-2 or BCDF (IL-6) were in fact measuring responses in normal cells.

It is worth noting here that residual normal B cells or peripheral blood B cells from normal volunteers do not represent the normal counterparts to B-CLL cells (for the purpose of a "normal control") because these cells are polyclonal in surface immunoglobulin light- and heavy-chain expression and do not carry the CD5 antigen, which (although being a T cell marker) is a hallmark of B-CLL cells. At the level of visual immunofluorescence there is only a small minority of CD5-positive B cells in the normal peripheral blood.53

B-CLL populations produced quantitatively similar levels of IgM after stimulation with TPA or TPA plus A23187 whether T cells were present or had been removed. In contrast, induction of Ig production from normal B cells by these same agents was strongly T cell dependent. These data suggest that stimulation of differentiation in B-CLL cells by direct activation of PKC is T independent, whereas in normal B cells, differentiation may be stimulated directly but remains largely dependent on helper effects (soluble or cell mediated) that are provided by T cells.

Elements of the signal transduction mechanism downstream of and including PKC seem to be intact in B-CLL because the cells from all patients responded appropriately to direct stimulation by phorbol ester. The rise in intracellular Ca2+ levels increased the potency of phorbol ester for inducing B-CLL cells to differentiate in 65% to 100% of the samples (depending on the parameters tested), thus indicating that that part of the protein phosphotransferase system that involves the second-messenger processing functions regularly in a coordinated process.

A recent publication56 suggested that PKC is also involved in IL-2–mediated signal transduction. By comparing the effects induced by agents that are known to directly activate PKC with the response seen after “physiological signal transduction” (upstream of PKC) with IL-2 and BCDF (IL-6) stimulation, we documented that the physiological ligands were unable to trigger differentiation in B-CLL cells, even though the signal transduction process evoked by extra-
cellular stimulants appears to be coordinated and effective in normal peripheral blood B cells.

Only Tac-positive normal B cells and not Tac-negative cells respond to IL-2 by differentiating into Ig-producing cells. B-CLL cells meet this precondition of expressing the IL-2 receptor. Thus, malignant B cell populations may spontaneously or after incubation with stimulants express the Tac antigen in the absence of a functional response to IL-2. This malfunction could be the result of an abnormality (chemical alteration?) of the IL-2 receptor or of a blockade of the signal between the IL-2 receptor and PKC.

Perri suggested that the defective expression of cell surface receptors for another B cell–directed lymphokine, B cell growth factor (BCGF), leads to an impaired responsiveness to BCGF by B-CLL cells. A similar defect might occur for the BCDF (IL-6) receptor. Alternatively, as proposed for IL-2, the processing of the initial signal given by BCDF–BCDF receptor interaction might be disturbed.

In the present study, we did not analyze the effects of anti-Ig antibodies of the B-CLL cells with regard to induced proliferation or differentiation. If RNA and DNA synthesis could be triggered by anti-Ig antibodies, then this might indicate that the signaling pathway of Slg receptors on B-CLL cells is in fact intact. Furthermore, because the phospholipid pathway is clearly not the only system used by lymphocyte receptors, this would suggest that different growth and differentiation factors use different messenger systems.

According to current dogma, members of a family of closely related guanosine triphosphate (GTP)-binding proteins (G proteins) are responsible for receptor-effector coupling in the transmembrane signaling. The activation of phospholipase C is G protein dependent. It has been shown that a variety of cell surface receptors that mediate growth, differentiation, secretion, etc, and that transmit their message by either the phosphatidylinositol-PKC system or through a second protein phosphotransferase system associated with the cAMP-dependent protein kinases, do so through a complex mechanism involving stimulatory (G) and inhibitory (Gi) activities of two different G proteins. Recent data suggest that the IL-2 receptor mediates signal transduction via a GTP-binding protein. This family of regulatory proteins that appear to represent crucial initial events for a variety of signal transduction reactions attracted much interest when it was described that GTP-binding proteins might be encoded by ras oncogenes. Further information on the G protein control of the transmembrane signaling linked to PKC activation will allow a more thorough evaluation of possible defects in the signal transduction that are possibly located in this part of the pathway.

In conclusion, the data demonstrate that commitment to the differentiation program can be initiated efficiently in B-CLL cells by phorbol ester and calcium ionophore acting together. The findings suggest that PKC activation by a direct signal delivered by TPA and A23187 leads to distinct responses in B-CLL cells that are characteristic of maturation towards plasma cells. The results indicate that the second-messenger system is not involved in any malfunction of the signal transduction system. On the other hand, a physiological response to the extracellular stimulants IL-2 and BCDF (IL-6) was not observed, which suggests that the molecular control of differentiation that affects the signal transduction at or immediately after ligand-receptor interaction might be impaired. It therefore appears that early signal transduction events (upstream of the activation of the key enzyme PKC in the cascade of signal transmission) might function incorrectly or not at all. The nature of this defect remains to be elucidated. Our data do not exclude that other growth or differentiation factors might use different messenger systems that might be intact in B-CLL cells.

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Analysis of signal transduction in B chronic lymphocytic leukemia cells

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