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**Research Article**

B cell chronic lymphocytic leukemia (CLL) cells appear to be arrested in their differentiation so that little immunoglobulin is secreted in most cases. To determine their capacity for further differentiation we stimulated cells from a series of 10 cases of CLL with a phorbol ester and assayed for production of immunoglobulin protein, accumulation of immunoglobulin mRNA, and alterations in cell surface markers. We found that cells from all cases were induced to secrete monoclonal immunoglobulin of the same heavy and light chain type as the surface membrane immunoglobulin type. Immunoglobulin secretion was preceded by a rapid increase in the levels of mRNA coding for IgM, predominantly the secretory form, mu s-mRNA, rather than the membrane form, mu m-mRNA. A similar selection of mu s- over mu m-mRNA is known to occur in plasma cells by a mechanism of differential processing of mRNA from a single mu-chain gene. Except for a decline in the expression of surface IgD, cell surface determinants remained unaffected both in terms of the percentage of positive cells and the relative number of sites per cell. In contrast to previous studies, these results indicate that CLL cells consistently retain the capacity to further differentiate toward plasma cells and secrete immunoglobulin. The immunoglobulin secretion is mediated, at least in part, by a developmentally regulated increment in mu s-mRNA.

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### In Vitro Enhancement of Immunoglobulin Gene Expression in Chronic Lymphocytic Leukemia

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**A**bstract. B cell chronic lymphocytic leukemia (CLL) cells appear to be arrested in their differentiation so that little immunoglobulin is secreted in most cases. To determine their capacity for further differentiation we stimulated cells from a series of 10 cases of CLL with a phorbol ester and assayed for production of immunoglobulin protein, accumulation of immunoglobulin mRNA, and alterations in cell surface markers. We found that cells from all cases were induced to secrete monoclonal immunoglobulin of the same heavy and light chain type as the surface membrane immunoglobulin type. Immunoglobulin secretion was preceded by a rapid increase in the levels of mRNA coding for IgM, predominantly the secretory form,  $\mu_s$ -mRNA, rather than the membrane form,  $\mu_m$ -mRNA. A similar selection of  $\mu_s$ - over  $\mu_m$ -mRNA is known to occur in plasma cells by a mechanism of differential processing of mRNA from a single  $\mu$ -chain gene. Except for a decline in the expression of surface IgD, cell surface determinants remained unaffected both in terms of the percentage of positive cells and the relative number of sites per cell. In contrast to previous studies, these results indicate that CLL cells consistently retain the capacity to further differentiate toward plasma cells and secrete immunoglobulin. The immunoglobulin secretion is mediated, at least in part, by a developmentally regulated increment in  $\mu_s$ -mRNA.

#### Introduction

Chronic lymphocytic leukemia (CLL)<sup>1</sup> is a slowly proliferative, monoclonal expansion of B lymphocytes in which the leukemia cells express surface membrane immunoglobulin (slg) but readily detectable paraprotein is not usually present in the serum (1). Although small amounts of monoclonal immunoglobulin may be secreted (2), CLL cells appear to be arrested in B cell differentiation before the stage of active immunoglobulin secretion usually associated with plasma cells. Despite the arrest of differentiation in vivo, in some instances leukemia cells can be manipulated in vitro to advance to more mature stages of differentiation (3-5). We and others have observed that some B cell precursor type acute lymphoblastic leukemias lacking both slg and cytoplasmic immunoglobulin (cIg) can produce both slg (6) and cIg (6, 7) when stimulated with the phorbol diester, 12-O tetradecanoylphorbol 13-acetate (TPA). This immunoglobulin production occurs in cells that have previously undergone rearrangement of immunoglobulin heavy and light chain genes in vivo and represents induction of the expression of functionally rearranged genes (6). Exposure of B-cell CLL to TPA has resulted in accumulation of cIg (8, 9) but has enhanced the secretion of immunoglobulin only in exceptional cases (10, 11). In a series of 17 cases of CLL treated with TPA, none was found to secrete immunoglobulin despite a sensitive detection assay (9).

In the present study we examined whether CLL cells could be induced to secrete immunoglobulin, and if the induction

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1. Abbreviations used in this paper: ABC, avidin-biotin complex;  $C_\mu$ ,  $\mu$ -chain gene; CLL, chronic lymphocytic leukemia; cIg, cytoplasmic immunoglobulin; ELISA, enzyme-linked immunoabsorbant assay;  $\mu_s$ -mRNA and  $\mu_m$ -mRNA,  $\mu$ -mRNA encoding the secretory and membrane forms of  $\mu$  chains, respectively;  $\mu$ -mRNA, messenger RNA encoding immunoglobulin  $\mu$  chain;  $\mu_m$ , DNA segment encoding the membrane anchoring piece in  $\mu_m$ -RNA; slg, surface membrane immunoglobulin; TPA, 12-O tetradecanoylphorbol 13-acetate.

affected immunoglobulin gene expression specifically at the mRNA level. To determine the latter point we exploited the fact that two mRNA coding for the  $\mu$ -immunoglobulin chain ( $\mu$ -mRNA) are selectively produced at two different stages of B cell differentiation. This choice is apparently mediated by an alternative recognition of distinct polyadenylation sites following the constant region of the  $\mu$ -chain gene ( $C\mu$ ). This allows for transcription of either the 2.4-kb  $\mu_s$ -mRNA coding for the secreted form of IgM or the larger 2.7-kb  $\mu_m$ -mRNA coding for surface membrane IgM from a single constant region gene (12). The latter message ( $\mu_m$ -mRNA) includes additional information from separated DNA axons encoding the membrane anchoring piece. Murine plasmacytomas and B cells actively secreting IgM contain more of the  $\mu_s$ -mRNA than  $\mu_m$ -mRNA, whereas more nearly equal amounts occur in low or nonsecreting B cells (13).

We now report that CLL cells, when treated with TPA, were in all cases induced to secrete immunoglobulin without a concomitant increase in the density of sIg. Further, the induction of immunoglobulin secretion was preceded by an increase in the level of mRNA-encoding IgM with a relative predominance of the  $\mu_s$ -mRNA form. The selective accumulation of  $\mu_s$ -mRNA followed by the secretion of immunoglobulin in CLL indicates that CLL cells can undergo plasmacytoid differentiation, which includes the developmentally regulated alternative processing of  $\mu$ -mRNA.

## Methods

**Cells and cultures.** Cell suspensions were prepared from blood and tissues involved by CLL from 10 patients as described (5). Cells were cultured at  $2 \times 10^6$ /ml in RPMI-1640 (Gibco Laboratories, Grand Island, NY) plus 10% fetal calf serum either with or without TPA at  $1.6 \times 10^{-8}$  M (Sigma Chemical Co., St. Louis, MO) in a humidified incubator at 37°C and 5% CO<sub>2</sub> (6).

**Immunostaining.** Cells were stained by indirect immunofluorescence and analyzed by a fluorescence-activated cell sorter (FACS II, Becton-Dickinson FACS Systems, Mountain View, CA) as described (6). The relative number of sites per cell of a surface membrane determinant was estimated by calculation of the mean channel number for the fluorescence intensity. Murine monoclonal antibodies included anti-immunoglobulin  $\gamma$ ,  $\alpha 1$ ,  $\alpha 2$ ,  $\delta$ ,  $\kappa$ ,  $\lambda$ , Leu 1 (14), HLA-DR (Becton-Dickinson & Co.), anti-immunoglobulin  $\mu$  (Bethesda Research Laboratories, Gaithersburg, MD), and B1 (15) (Coulter Electronics Inc., Hialeah, FL). Cytoplasmic immunoglobulin was identified on acetone-fixed (24°C, 5 min) cytocentrifuge preparations using the murine monoclonal antibodies to human immunoglobulins followed by the avidin-biotin complex (ABC) peroxidase technique (Vector Laboratories, Inc., Burlingame, CA) (16).

**Enzyme-linked immunoabsorbant assay (ELISA).** Cell-free culture supernatants (50–200  $\mu$ l) were added in duplicate to 96-well NUNC immunoplates (No. 2-39454) precoated with 200  $\mu$ l of chain-specific, affinity-purified goat anti-human  $\mu$ ,  $\gamma$ ,  $\kappa$ , or  $\lambda$  (1.6  $\mu$ g/ml; Tago Inc., Burlingame, CA). Standards of known quantities of the various human heavy and light chains were added as well. After incubation at room temperature for 2–4 h or overnight at 4°C, the ELISA was completed using the ABC technique (16). Briefly, biotinylated goat anti-human  $\mu$ ,  $\gamma$ ,  $\kappa$ , or  $\lambda$  (1:350 dilution; Tago Inc.) was added to the plates followed by the ABC reagent (avidin-DH complexed to biotinylated horseradish

peroxidase-H, Vector Laboratories). Color was developed by addition of *o*-phenylenediamine (Aldrich Chemical Co., Inc., Milwaukee, WI) in the presence of hydrogen peroxide. The reaction was terminated by addition of 8 N sulfuric acid and optical densities determined at 492 nm using a Titertek multiscan photometer (Flow Laboratories, Inc., McLean, VA). Nanogram values were determined by use of a standard curve.

**Nucleoside incorporation.** 100,000 cells were pulsed (37°C, 4 h) with 1  $\mu$ Ci [methyl-<sup>3</sup>H]thymidine (sp act 47 Ci/mmol, New England Nuclear, Boston, MA). Samples were filtered, precipitated by trichloroacetic acid, and counted in Aquassure (New England Nuclear) on a scintillation counter.

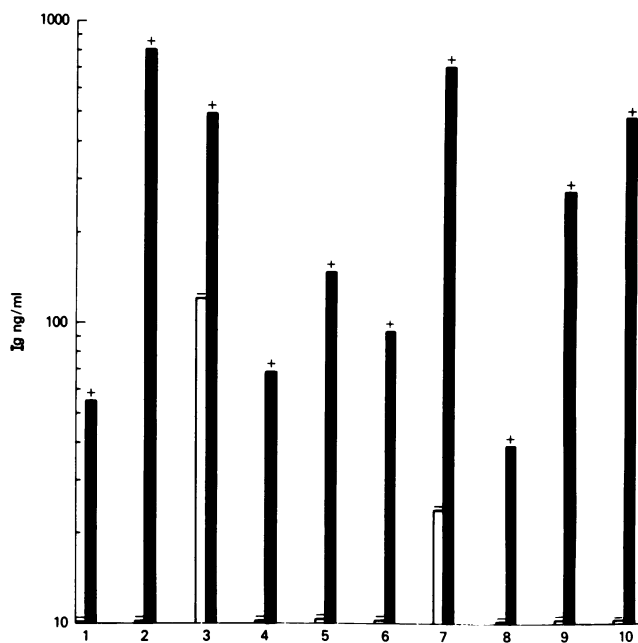
**Determination of immunoglobulin RNAs.** To assess the abundance of mRNA coding for immunoglobulin, several approaches were undertaken. Firstly, total cellular RNA was extracted at serial time points from the lymphocytes by suspending the cells in 4 M guanidine-thiocyanate, disrupting the cells with a polytron (Brinkmann Instruments, Inc., Westbury, NY), and pelleting the RNA through a 5.7-M CsCl<sub>2</sub> gradient (17). The relative abundance of immunoglobulin mRNA as a proportion of total cellular RNA was determined by immobilizing, in duplicate, 10, 5, and 2.5  $\mu$ g of the RNA directly onto nitrocellulose paper (18). Hybridization to these total cellular RNA dot blots was carried out as previously reported with <sup>32</sup>P nick-translated genomic DNA probes of the constant regions of C $\mu$  and kappa chain and the C $\mu$  membrane anchoring piece ( $\mu_m$ ) (19) (Fig. 4). Relative changes in immunoglobulin RNAs were quantified by determining the counts per minute hybridized to these duplicate RNA dot blots. Secondly, the relative proportion of the 2.7-kb  $\mu_m$ -mRNA to 2.4-kb  $\mu_s$ -mRNA was determined by Northern blot analysis in which 10  $\mu$ g of total cellular RNA was electrophoresed through a formaldehyde gel, transferred to nitrocellulose, and hybridized with the <sup>32</sup>P nick-translated C $\mu$  DNA probe (18). Finally, the relative abundance of Ig-mRNA per cell was assayed by the cytoplasmic dot blot method described by White and Bancroft (20). Detergent-solubilized cytoplasm from a standardized number of cells was applied in various dilutions to the wells of a 96-hole suction manifold apparatus (Hybridot, Bethesda Research Laboratories) containing a nitrocellulose filter. All filters were baked (80°C, 90 min, in vacuo) and hybridized with either the C $\mu$ -, C $\kappa$ -, or  $\mu_m$ -DNA probes. For the latter two methods, autoradiograms were developed and relative levels of specific RNA were compared within the linear dose response of the film by scanning densitometry.

## Results

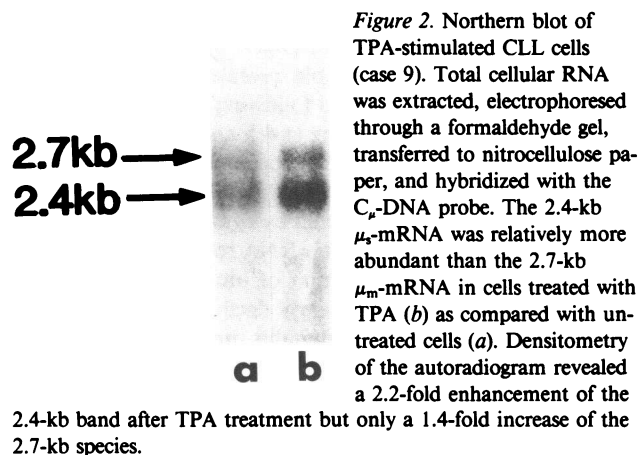
When exposed to TPA, CLL cells underwent profound morphologic alterations by 3 d and progressed from their small lymphocyte appearance to assume plasmacytoid and immunoblastic features with multinucleation and prominent nucleoli. Although an increase in DNA synthesis was detected (10–30  $\times 10^3$  counts/10<sup>6</sup> cells per min for TPA-exposed cells as compared with 10<sup>3</sup> counts for control cells), TPA treatment did not result in cell proliferation (no increase in cell count over 3 d). At 2 d of exposure to TPA, there was a marked increase of intracytoplasmic immunoglobulin of monoclonal heavy and light chain type identical to the corresponding surface immunoglobulin isotypes. Parallel cultures of untreated cells had neither morphologic alterations nor increased intracytoplasmic immunoglobulin. In all 10 cases, monoclonal immunoglobulin of

a single heavy and light chain type identical to the sIg isotype was detected in the culture supernatants of TPA-stimulated cells by 3 d (Fig. 1). In all but two cases, immunoglobulin was undetectable in supernatants of untreated cells, and even in the two exceptional cases (cases 3 and 7) a marked enhancement of immunoglobulin secretion was obtained with exposure to TPA. TPA-stimulated cells were analyzed daily for 3 d for alterations in cell surface determinants with a battery of monoclonal antibodies (Leu1, HLA-DR, B1, and anti-surface immunoglobulin  $\mu$ ,  $\delta$ ,  $\kappa$ , and  $\lambda$ ). Initially, cells from all cases expressed monoclonal sIg, Leu1, HLA-DR, and B1. Of these, only surface IgD deviated more than 15% from control values with respect to the percentage of cells stained (38% of control at 3 d). The relative number of sites per cell (mean fluorescence intensity) of all surface antigens, including sIgD, were not significantly affected during 3 d of exposure to TPA (<15% deviation from control).

We then questioned if the induction of immunoglobulin secretion was associated with an increase in mRNA coding for immunoglobulin and, if so, was there a selective increase in the 2.4-kb mRNA-encoding secretory  $C_{\mu}$  in a manner suggesting plasmacytoid differentiation? Total cellular RNA was extracted from cells that were either untreated or exposed to TPA (case 9). Northern blots of this RNA, when hybridized with the  $C_{\mu}$

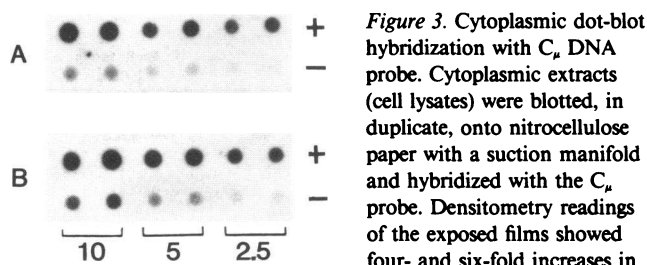


**Figure 1.** Immunoglobulin  $\mu$  chain secretion by CLL cells. Cells ( $2 \times 10^6$ /ml) from each of 10 cases were cultured for 3 d. Supernatants were collected and immunoglobulins were quantified by an ELISA. In all cases, immunoglobulin secretion was increased by the addition of  $1.6 \times 10^{-8}$  M TPA (solid bars). Secreted monoclonal heavy and light chain isotypes corresponded to the surface Ig in all instances. Parallel cultures in which TPA was omitted are displayed as open bars.



probe, revealed an increase in  $\mu$ -mRNA that was predominantly the 2.4-kb  $\mu_s$ -mRNA as opposed to the 2.7-kb  $\mu_m$ -mRNA species in cells treated with TPA (Fig. 2). After 18 h the TPA-treated cells showed a 2.2-fold enhancement of the 2.4-kb band whereas the 2.7-kb band increased only 1.4-fold above the untreated control, as determined by densitometry. This Northern blot analysis used a standard quantity of total RNA in each lane and thus measured changes in  $\mu$ -mRNA that were relative to the total amount of RNA present. Because the total cellular RNA recovered at 24 h was twice as much in induced as in unstimulated cells we next chose to examine the increment in  $\mu$ -mRNA on a per cell basis. To accomplish this, cytoplasm was detergent-solubilized from equal numbers of cells cultured in the presence or absence of TPA at time points of 0, 4, 8, and 24 h. These cytoplasmic dot blots were hybridized with the  $C_{\mu}$  probe and the autoradiograms, when scanned by densitometry, revealed a maximum increase in  $\mu$ -mRNA at 24 h that was four- and six-fold greater than initial levels in cases 9 and 10, respectively (Fig. 3).

To more closely examine the dynamics of induction of immunoglobulin mRNA, total cellular RNA was extracted at serial time points during continuous exposure to TPA. This was an-

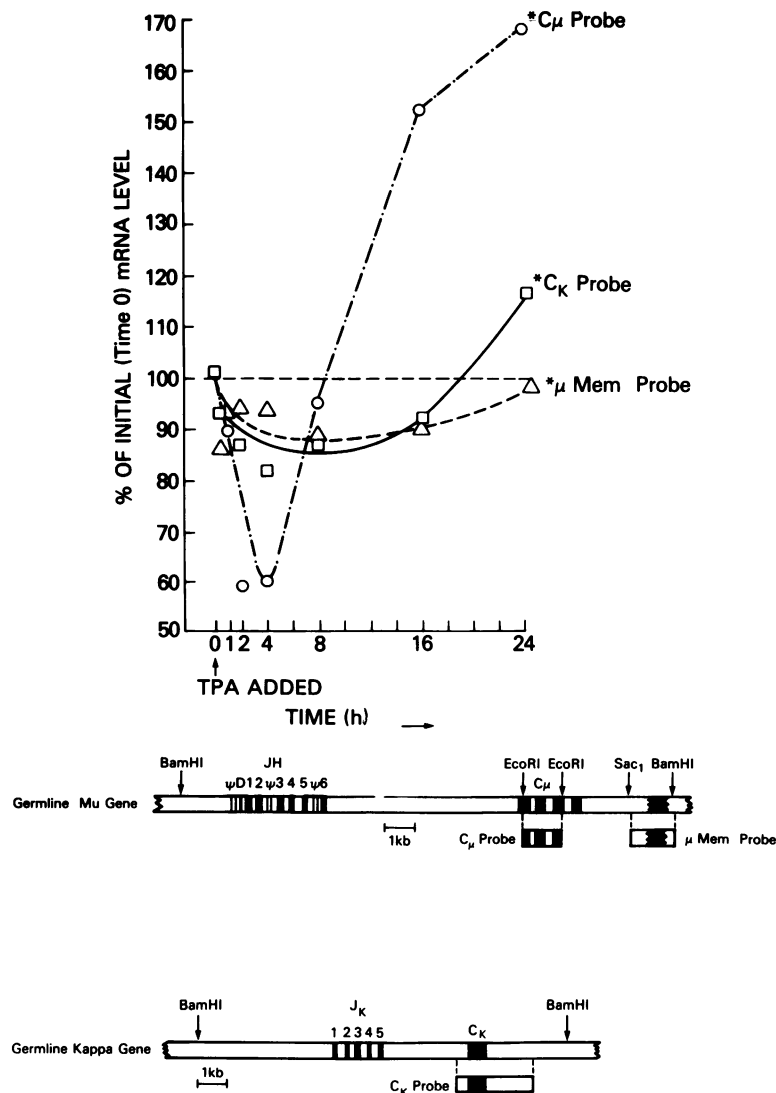


**Figure 3.** Cytoplasmic dot-blot hybridization with  $C_{\mu}$  DNA probe. Cytoplasmic extracts (cell lysates) were blotted, in duplicate, onto nitrocellulose paper with a suction manifold and hybridized with the  $C_{\mu}$  probe. Densitometry readings of the exposed films showed four- and six-fold increases in  $\mu$ -mRNA levels when cases 10 (A) and 9 (B), respectively, were treated with TPA for 24 h (+) as compared with similarly cultured and prepared cells that did not receive TPA (-). The numbers at the bottom indicate the number of cells ( $\times 10^{-5}$ ) applied in the blotted sample.

alyzed by immobilizing, in duplicate, 10, 5, and 2.5  $\mu\text{g}$  of total cellular RNA onto nitrocellulose paper. Scintillation counting of each hybridized RNA sample revealed that the level of  $\mu$ -mRNA was initially depressed following the addition of TPA but abruptly increased beginning at 4 h and reached a peak 24 h after TPA (Fig. 4). The nearly twofold increase in  $\mu$ -mRNA detected in these total cellular RNA dot blots was in accordance with the fourfold increase detected by cytoplasmic dot-blot hybridization, since the total cellular RNA recovered at 24 h from TPA-treated cells was double that of untreated cells. Kappa-mRNA levels also showed an early decline, but gradually increased above initial levels. During this time course,  $\mu_m$ -mRNA never exceeded initial base-line levels, independently confirming that the increment in  $\mu$ -mRNA was almost exclusively the  $\mu_s$ -mRNA secreted form.

## Discussion

We have demonstrated the induction of immunoglobulin secretion in all cases of CLL tested in this study. These studies confirm previously reported observations of intracellular immunoglobulin production and secretion in some cases of CLL when stimulated by TPA (8–11), mitogens (21), or allogeneic T cells (22). The rather uniform immunoglobulin secretion we have observed in a series of cases of CLL contrasts with previous studies and may reflect either the heterogeneity of the disease and the differential capacities of CLL to respond to stimulation in vitro (21) or the differences in the culture protocols and sensitivity of the detection assays. One potential benefit of the capability of CLL to consistently differentiate into efficient secretory cells in vitro is to provide a source of monoclonal im-



**Figure 4.** Kinetics of  $\mu$ -mRNA accumulation in TPA-treated CLL cells (case 9). At serial time points following exposure to TPA, total RNA was extracted with guanidine-thiocyanate and 10, 5, and 2.5  $\mu\text{g}$  of RNA was blotted onto nitrocellulose filters and hybridized with the  $^{32}\text{P}$  nick-translated DNA probes of C $\mu$ ,  $\mu$ -m, and C $\kappa$  (schematically represented in the lower diagram). The duplicate dots were then cut out and scintillation counted. Total  $\mu$ -mRNA (C $\mu$  probe) declined initially but rapidly increased between 4 and 24 h.  $\kappa$ -mRNA also declined initially and increased slightly by 24 h. In contrast,  $\mu_m$ -mRNA did not show an increase above initial levels.

munoglobulin sufficient for immunization and production of hetero-antiidiotypic antibodies. We have found that several milligrams of secreted monoclonal immunoglobulin can be harvested from culture supernatants of TPA-induced CLL cells, purified by affinity chromatography, and used for the immunization and screening required to produce monoclonal (hybridoma) antiidiotypic antibody (unpublished results). Antiidiotypic antibodies may be valuable for both monitoring and passive immunotherapy of some B cell malignancies (23, 24).

The many changes exhibited by TPA-stimulated CLL cells suggest differentiation toward the plasma cell stage. A concurrent loss of sIgD and induction of immunoglobulin secretion occurs in normal B cells when stimulated by polyclonal B-cell activators (25). Furthermore, the stimulated CLL cells acquired morphological features of plasma cells and immunoblasts and accumulated abundant intracellular immunoglobulin. The mechanism by which TPA induces these changes is not known but may be initiated by calcium-dependent protein-kinase C when activated by exposure to TPA (26–29). The IgM secreted by induced cells is a product whose appearance follows a four–six-fold increment in  $\mu$ -mRNA and thus appears to be regulated, at least in part, at a pretranslational level. Furthermore, the fact that the increase was selectively in the secreted and not the membrane form of  $\mu$ -mRNA supports the hypothesis that plasmacytoid differentiation was induced by TPA. While the exact mechanism of this accumulated  $\mu$ -mRNA is uncertain, its rapid increase argues that an enhancement of transcription is occurring. That considerable  $\mu_s$ -mRNA existed in the cells before stimulation may indicate that translational and posttranslational events (30, 31) are also affecting this immunoglobulin production. Such mechanisms play a significant role in immunoglobulin induction in murine B cells (32) and in the murine B cell line, BCL-1 (13).

The present studies demonstrate that CLL cells retain the capacity to advance beyond the stage at which they are arrested in vivo and that the induced changes are likely differentiation phenomena. These differentiation events include a selective increase in the  $\mu_s$  relative to the  $\mu_m$  form of  $\mu$ -mRNA during the maturation of CLL cells. A monoclonal expansion of neoplastic B cells at a specific stage of differentiation, such as CLL, provides an opportunity to dissect the mechanisms that regulate immunoglobulin gene expression. Processes uncovered by the study of CLL cells in vitro may provide insights into the signals that prevent differentiation in vivo.

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