Heterogeneity of the Response of Chronic Lymphocytic Leukemia Cells to Phorbol Ester

By Jun Okamura, Erwin W. Gelfand, and Michelle Letarte

The ability of the tumor promoter 12-0-tetradecanoylphorbol 13-acetate (TPA) to induce differentiation of leukemic cells was studied in 10 cases of chronic lymphocytic leukemia (CLL). An increase in modal volume and an enhancement of the capacity of the leukemic cells to stimulate in mixed lymphocyte reaction (MLR) was seen in the majority of cases. A significant increase in Ia expression was observed upon culture of leukemic cells with TPA in 6 of the 10 cases; 5 of these cases also showed an induction of cytoplasmic IgM production. Correlations between the phenotypic markers of the leukemic cells and their ability to respond to TPA were evaluated. CLL cells with low amounts of surface Ig, a volume $\leq$165 fl, and relatively low Ia expression responded well to TPA. Cells with bright surface Ig, a volume $\geq$178 fl, and elevated amounts of Ia responded poorly to TPA. These results suggest that differences in the response of B leukemic cells to TPA reflect the underlying heterogeneity of the leukemic cells and might be correlated with their stage of maturation.

Cell Separation, Volume, and Marker Studies

Peripheral blood was obtained from 9 patients by leukopheresis, while a bone marrow sample was obtained from patient PA. Mononuclear cells were separated by Ficoll-Hypaque gradient centrifugation and washed with phosphate-buffered saline (PBS, pH 7.25). Cell size distribution was determined using a Coulter model ZBI counter linked to Coulter Channelizer. The modal volume was estimated from calibration against polystyrene microspheres of diameter 10 and 19 $\mu$m (Coulter Electronics, Hialeah, Fla.) and is expressed in fentoliters (fl).

The percentage of T cells was estimated by the sheep erythrocyte rosetting assay (E-rosette) performed as described previously. Surface Ig was detected using fluoresceine isothiocyanate (FITC) conjugated goat anti-human Ig, polyvalent or specific for $\mu$, $\alpha$, $\gamma$, $\kappa$, or $\lambda$ chains (Meloy Laboratories, Springfield, Va.). Cytoplasmic Ig was detected using FITC-conjugated goat anti-human $\mu$ chain after the parental myeloma cell line P3/X63 Ag8 (P3), followed by incubation with FITC-goat F(ab')2 anti-mouse Ig, polyvalent or specific for $\mu$, $\alpha$, $\gamma$, $\kappa$, or $\lambda$. Cell viability was assessed by trypan blue dye exclusion and cell numbers were estimated with the Coulter Counter.

Cell Cultures

CLL cells were cultured for 1–7 days at a concentration of $10^7$/ml, with or without TPA, in Falcon plastic culture flasks at 37$^\circ$C, in 5% CO$_2$ humidified atmosphere, in RPMI 1640 medium supplemented with penicillin, streptomycin, and 10% heat-inactivated fetal calf serum (FCS). Ten percent heat-inactivated human AB serum was substituted for FCS in 3 cases (PA, HA, RA). Cultures were set up in triplicate or duplicate except in cases NA and VE, where only one could be set up. Following culture, cells were harvested and washed 3 times with PBS. Cell viability was assessed by trypan blue dye exclusion and cell numbers were estimated with the Coulter Counter.

TPA (P-L Biochemicals, Milwaukee, Wisc.) was dissolved in acetone at a concentration of $1.6 \times 10^{-4}$ M and kept at $-20^\circ$C. It was diluted directly into culture medium at the time of the experiment. TPA was used at a final concentration of $1.6 \times 10^{-7}$ M.

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**RESULTS**

**Heterogeneity of Leukemic Cells**

In initial studies, the phenotypic expression of markers was investigated on the 10 CLL cases. All leukemic cells were derived from patients with elevated WBC counts, varying from 45 to 286 \( \times 10^9 \) cells/liter (Table 1), and thus the mononuclear fraction consisted almost exclusively of leukemic cells. An average of 89% of the mononuclear cells were Ia positive, while only 4% on average formed E-rosettes.

Surface Ig could be detected on all but one of the patients (Table 2). The majority of mononuclear cells expressed surface Ig, although the percentage of positive cells could not be accurately estimated in several cases because of the faint staining observed (Table 2). Cells reactive with polyvalent anti-human Ig were also reactive with anti-\( \mu \), and either anti-\( \kappa \) or anti-\( \lambda \) chains. Three patients (KE, HA, and AL) showed bright surface Ig staining; patients AL and HA were diagnosed as having prolymphocytic leukemia. The percentage of CLL cells bearing cytoplasmic Ig was less than 0.5%.

The modal volume of leukemic cells showed a wide range of variation from 110 to 218 fl (Table 2). A similar range has been reported previously for CLL cells, with prolymphocytic leukemia having a larger volume than the classical type of CLL.

The amount of Ia estimated at the surface of the leukemic cells varied from 1.4 to 10.8 \( \times 10^5 \) molecules per cell (Table 2). The heterogeneity of Ia expression on CLL cells is clearly illustrated in Fig. 1.
The amount of Ia present on $5 \times 10^8$ leukemic cells was estimated in the CRIA using saturating amounts of 21w4 antibody and 500 ng of $^{125}$I-RAM-Fc. For each case, saturation was established using concentrations of 21w4 culture supernatant varying from 1.5% to 50% during the binding reaction. All experiments were done in triplicate and results are expressed as the mean ± SD of the numbers of molecules of RAM-Fc bound per cell.

correlation was observed between the amount of Ia and the estimated surface area. The Ia density, calculated as the approximate number of molecules per unit of surface area, was 4-5 times higher for cases RA, HA, and AL than for cases KL, NA, PA, and KE, while the other 3 cases were of intermediate value. Thus, it appears that increased amounts of Ia on CLL cells are associated with an increased density of Ia due either to augmented synthesis of the glycoproteins or to greater availability for antibody binding.

Effects of TPA on CLL Cells

The effects of in vitro culture with the phorbol ester TPA were measured on leukemic cells derived from the 10 patients. Prior to culture, cell viability was always greater than 95%. The average cell viability following culture with or without TPA was 97%, 89%, and 63% on days 1, 3, and 7, respectively. Cell recovery was usually greater than 80% on days 1 and 3. Several changes in the properties of CLL cells were induced by TPA, which can be summarized as follows.

Cell volume. Figure 2 demonstrates the effects of TPA on the modal volume of CLL cells. After in vitro culture for 1 day in control medium, a minimal increase in cell volume (mean of 4%) was observed in 9 patients. In contrast, TPA induced an average increase of 19% in the volume of the same cells after 1 day of culture. The size of the CLL cells remained enlarged even after 6 or 7 days of culture with TPA.

Surface Ig. The percentage of surface Ig$^-$ cells could not always be followed during culture due to faint fluorescent staining. The 3 patients with bright surface Ig at the initiation of culture (Table 2) showed no demonstrable change in the percentage or the intensity of surface Ig$^-$ cells during the 3-day culture with or without TPA. In contrast, surface Ig$^-$ cells decreased in number and became undetectable after 3 days culture with TPA for patients MC and PA; no decrease in surface Ig$^-$ cells was observed in the control cultures.

Cytoplasmic Ig. As demonstrated in Table 2, CLL cells cultured with TPA became strongly positive for cytoplasmic Ig in 5 of the 10 cases. The percentage of cells staining for cytoplasmic Ig varied from 21% to 92% between day 1 and day 7 of culture with an average of 71%. Cytoplasmic Ig remained negative (<1%) in all the control cultures. No induction of cytoplasmic Ig could be observed in the other 5 cases, which included the 3 cases with initial bright surface Ig staining (Table 2).

Surface Ia. There was a significant increase in the amount of Ia expressed on CLL cells cultured with or without TPA (Fig. 3). The TPA-treated cells expressed significantly higher amounts of Ia than control (FCS) cells in 7 patients ($p < 0.05$, Fig. 3). The percent increase seen in TPA-treated cultures ranged from 46% to 336%, and the actual amount of Ia observed after TPA treatment varied from 5.5 to 22.5 $\times 10^5$ molecules/cell. The stimulation of Ia expression on CLL cells by TPA was seen with concentrations ranging from $10^{-6}$ to $10^{-10}$ M and appeared optimal between $10^{-7}$ and $10^{-9}$ M. The increase seen following culture with FCS alone is likely due to serum factors that are stimulatory for B cells. Decreasing the FCS concentration to 5% or 2%, or substituting human AB serum for FCS, did not significantly diminish the increase in Ia expression induced by the culture conditions. However, the amount of Ia on control (FCS)
Fig. 3. Increase in Ia expression on CLI cells following in vitro culture. Leukemic cells were cultured with TPA (solid bars) or without TPA (hatched bars) as in Fig. 2. The amount of Ia present prior to culture and following culture with or without TPA was determined as shown in Fig. 1. The percent increase in Ia expression was calculated as (Molecules RAM – Fc bound per cultured CLI cell/Molecules RAM – Fc bound per CLI cell prior to culture – 1) x 100. Most cultures were done in duplicate or triplicate flasks; values shown represent the mean ± SD of the percent increase in Ia.

cultures tended to return after 7 days in culture to the level seen on fresh cells, whereas it remained elevated on TPA-treated cells.

When results were calculated as the difference in the percent increase in Ia between the TPA-treated groups and the control groups, 6 cases were found to respond well to TPA while 4 others did not (Table 2). All six cases that responded to TPA also were noted to demonstrate faint surface Ig, relatively low Ia, and a cell volume less than 166 fl. In addition, in 5 of these 6 cases, induction of cytoplasmic Ig was observed in response to TPA. The 4 cases not responding to TPA with a large increase in Ia remained negative for cytoplasmic Ig. Three of these 4 cases were noted to express bright staining for surface Ig, abundant Ia, and a cell volume larger than 178 fl. However, exceptions to these groups were noted (Table 2).

**Mixed lymphocyte reaction.** As shown in Table 3, leukemic cells from 6 of 8 cases cultured in the absence of TPA were stimulatory in MLR with at least one of the normal responders tested. These results were not different from those observed with the fresh CLL cells (data not shown). Comparison with control MLR (responder, versus responder2) suggested that the majority of CLL cases showed good stimulatory capacity even when low concentrations of stimulator cells were used. These results contrast with a previous report suggesting that CLL cells are poor stimulators in MLR. No correlation between the amount of Ia present on CLL cells (measured with 21w4 monoclonal antibody) and the ability to stimulate in MLR was observed.

Following incubation with TPA, the CLL cells showed an enhanced stimulatory capacity in MLR when compared to CLL cells cultured for the same

<p>| Table 3. MLR Stimulatory Capacity of CLL Cells After Culture With TPA |
|----------------|----------------|----------------------|----------------|</p>
<table>
<thead>
<tr>
<th>Patient</th>
<th>Responder</th>
<th>3H-Thymidine Incorporation (cpm)</th>
<th>Ratio of Stimulatory Capacities TPA /TPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>KL</td>
<td>R</td>
<td>586 ± 173</td>
<td>25,366 ± 4,714</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>588 ± 143</td>
<td>28,107 ± 445</td>
</tr>
<tr>
<td>PA</td>
<td>R</td>
<td>298 ± 107</td>
<td>2,000 ± 335</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>9,817 ± 2,227</td>
<td>8,061 ± 1,154</td>
</tr>
<tr>
<td>KE</td>
<td>R</td>
<td>849 ± 90</td>
<td>2,239 ± 308</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>7,899 ± 727</td>
<td>4,876 ± 279</td>
</tr>
<tr>
<td>VE</td>
<td>R</td>
<td>634 ± 275</td>
<td>6,586 ± 439</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>6,512 ± 157</td>
<td>27,348 ± 2,186</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>6,872 ± 501</td>
<td>39,349 ± 5,130</td>
</tr>
<tr>
<td>MC</td>
<td>R</td>
<td>13,595 ± 4,398</td>
<td>104,915 ± 14,552</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>66,289 ± 7,763</td>
<td>103,111 ± 9,151</td>
</tr>
<tr>
<td>RA</td>
<td>R</td>
<td>4,884 ± 1,827</td>
<td>7,059 ± 963</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>2,140 ± 218</td>
<td>6,794 ± 759</td>
</tr>
<tr>
<td>AL</td>
<td>R</td>
<td>16,403 ± 3,463</td>
<td>32,553 ± 3,136</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>4,341 ± 1,117</td>
<td>25,740 ± 2,402</td>
</tr>
</tbody>
</table>

CLL cells were incubated with or without TPA for 16 hr except for AL cells, which were incubated for 64 hr. CLL cells were then used as stimulators in MLR as described under Materials and Methods. All assays were done with 3 stimulator cell concentrations (2.5, 5, and 10 x 10⁶ cells/ml). The data obtained with 5 x 10⁹ cells/ml are shown in the table.
period in medium plus FCS (Table 3). The degree of augmentation was more prominent at stimulator cell concentrations of 1 and $5 \times 10^7$ cells/ml than at $10 \times 10^9$ cells/ml. With all CLL cases studied, a significant increase in stimulatory capacity by TPA was seen with at least one of the responders. The data in Table 3 indicate that TPA can enhance the ability of CLL cells to stimulate in the MLR, but to variable extents. No correlation was observed between the augmented ability of CLL cells to stimulate in MLR and the increased expression of Ia on the leukemic cells following induction by TPA (compare Fig. 3 and Table 3).

**DISCUSSION**

The tumor promoter TPA is an active modulator of differentiation of both myelogenous and lymphocytic leukemias. Myeloid cells derived from patients with chronic or acute leukemia or acute myelomonocytic leukemia can be converted by in vitro culture with TPA into macrophage-like cells. The promyelocytic leukemia line HL-60 could also be induced to differentiate into monocyte-macrophage cells producing colony-stimulating activity. The leukemic T-cell line Molt-3 responded to TPA by a sequence of events characteristic of the maturation of prethymic precursor T cells, including the loss of terminal deoxynucleotidyl transferase, loss of the ability to proliferate, and the appearance of E-rosette receptors.

CLL cells could also be induced to mature along the B lineage by TPA as shown by characteristic morphological changes and by conversion of surface Ig$^+$ cells into cytoplasmic Ig$^+$ cells. Furthermore, we have shown that TPA can induce CLL cells to secrete IgM, can increase the amount of Ia expressed on the surface of the blast cells, and can augment the ability of CLL cells to stimulate in the MLR.

The present study confirms that TPA has the particular property of triggering the maturation of CLL cells. Of the 10 cases analyzed, 6 were shown to significantly increase the amount of cell surface Ia in response to TPA. Of these 6 cases, 5 could be induced to produce cytoplasmic Ig (Table 2). These TPA-responsive cases demonstrated the following characteristics: low surface Ig, no detectable cytoplasmic Ig, a volume between 110 and 165 fl, and amounts of Ia varying between 1.4 and $4.2 \times 10^9$ molecules/cell. The 4 cases not responding to TPA with a large increase in surface Ia remained cytoplasmic Ig-negative and continued to express surface Ig. Three of these four cases were comprised of cells with a volume greater than 178 fl and with high amounts of surface Ig. Only one exception was found with respect to the amount of Ia and the response to TPA: KE cells, which carried low amounts of Ia ($2.5 \times 10^7$ molecules/cell), did not respond to TPA. Otherwise, all patients whose cells expressed less than $4.2 \times 10^9$ Ia molecules/cell responded to TPA, while the 3 cases with greater than $7.8 \times 10^9$ molecules/cell did not respond.

In the present study, the ability to respond to TPA thus appears to segregate into two distinct groups. The nonresponsive group comprises patients whose leukemic cells are larger and with a higher density of surface Ig and surface Ia than the cells responding to TPA. Included in this group are two patients clinically diagnosed as having prolymphocytic leukemia. In the second group, the leukemic cells could be altered by TPA in a number of ways that mimic normal B-cell maturation. Delineation of the stages of B-cell maturation is somewhat limited when compared to stages identified for T lymphocytes. Parameters characteristic of B-cell maturation include morphological changes, progressive reduction of slg, appearance of cytoplasmic Ig (with heavy and light chain reactivities) and Ig secretion. Overall, the patients responding to TPA initially carry the phenotypic markers of classical CLL, i.e., low surface Ig, relatively low Ia, and small cell volume. They respond to TPA with an increase in Ia and cell volume, a diminution in slg staining intensity, express cytoplasmic IgM, and, in the one case described previously, secrete IgM. It has been reported that myelogenous leukemic cells representing early stages of differentiation were resistant to induction by TPA, whereas cell lines derived from later stages of maturation were induced to macrophage-like cells by TPA. It is possible that the response of CLL cells to TPA may similarly reflect the stage of maturation, more immature cells being less or nonresponsive.

The increase in volume of the CLL cells induced by TPA (Fig. 2) is in contrast to the previously reported decrease in volume in T-lymphoblastoid cell lines treated with TPA or in Friend leukemia cells treated with dimethyl sulfoxide. Since only 30-min exposure to TPA was sufficient to cause an increase in cell volume, this change may not be indicative of a differentiation step. Cell volume regulation in lymphoid cells appears to be mediated by ion fluxes, is calcium-dependent, and appears to involve calmodulin. The generalized membrane perturbation that is induced by TPA may alter the normal sequence of events that regulates cell volume, thus leading to a significant increase in the cell volume in isotonic media.

The mechanism for the increase in Ia expression on cultured CLL cells (Fig. 3) has similarly not been clarified. The increase in Ia as assessed in the CRIA may represent an increase in de novo synthesis. Alternatively, the augmentation following culture with TPA may be due to an increase in the number of available Ia antigenic sites. FCS is known to be mitogenic for lymphocytes and to result in changes in surface antigen expression.
EFFECTS OF TPA ON CLL CELLS

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revealed that DNA synthesis remained extremely low in CLL cells cultured with or without TPA despite the increase in Ia expression. The mechanism responsible for the augmentation in Ia might be different for FCS and TPA. In the presence of FCS, Ia expression returned to resting levels by day 7 of culture, while remaining elevated in the presence of TPA. Further, culturing CLL cells with FCS did not result in changes in the other parameters measured.

There has been some controversy in the past concerning the ability of CLL cells to stimulate in an allogeneic MLR.10,21,22 Certainly, on the basis of B-cell numbers, CLL cells are inferior to normal B cells.10 Nevertheless, 6 of 8 of our patients were effective stimulators for at least one of the two responders used in each experiment, even when used at low concentrations (5 x 104 stimulator cells/culture, 2.5 x 105 cells/ml). Wolos and Davey previously suggested a direct correlation between Ia and MLR stimulatory capacity.22 Certainly, on the basis of B-cell ontogeny concerning the ability of CLL cells to stimulate in an autologous and allogeneic MLR. As seen from the data in Fig. 3 and Table 3, there was no direct correlation between the enhanced capacity to stimulate in the MLR and the increase in Ia expression. The enhancement by TPA may be explained by membrane perturbation or conformational changes in the Ia glycoproteins; alternatively, TPA may cause the induction or alteration of determinants other than Ia, which are stimulatory in the MLR.

The present report confirms and extends the data of the effects of TPA on CLL cells. Several lines of evidence suggest that the initial site of action of TPA is the plasma membrane and indeed, saturable receptors for phorbol esters have been identified in cell membranes.27-28 The identification of a number of changes in phenotypic markers induced by TPA in one group of CLL cells but not in the other may suggest the presence of TPA receptors in the former but not the latter group. However, the ability of TPA to augment MLR stimulatory capacity in cells from both groups could indicate that TPA receptors are also present in the nonresponding group. Alternatively, the effect of MLR stimulation may be due to a nonspecific interaction of lipophilic TPA with the plasma membrane. More likely, the changes induced by TPA in the cells from the two groups reflect their stage of maturation, as seen with cells of granulocyte lineage,13 although differences in affinity or number of phorbol ester receptors cannot be discounted at present. Nevertheless, the study of the ability of leukemic cells to respond to TPA has revealed a previously unrecognized heterogeneity among CLL cells. Furthermore, this type of study may help in identifying the sequence of events in normal B-cell ontogeny.

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