Induction of Proliferation of B Prolymphocytic Leukemia Cells by Phorbol Ester and Native or Recombinant Interferon-γ

By R.W. Sauerwein, W.G.J. van der Meer, and L.A. Aarden

Phorbol ester phorbol myristate acetate (PMA) induces proliferation in nonmalignant human B cells and B cells from a patient with B prolymphocytic leukemia (B-PLL). Mitogen-free T cell–derived conditioned medium acts synergistically with PMA in inducing proliferation of B-PLL cells but does not enhance the PMA-stimulated outgrowth of nonmalignant B cells. Interleukin 2 (IL-2) has no effect on the outgrowth of B-PLL cells, and monoclonal antibodies against the IL-2 receptor do not influence the response to PMA and conditioned medium. Recombinant interferon-γ (IFN-γ), in contrast, is a potent enhancer of PMA-induced proliferation of B-PLL cells. With gel filtration techniques and with the use of anti–IFN-γ antibodies, it is shown that IFN-γ in the conditioned medium is responsible for the observed increase in B-PLL cell proliferation. Preincubation of B-PLL cells with IFN-γ induces responsiveness to PMA, whereas IFN-γ alone had no effect on these cells when pretreated with PMA. The combined data show that, in the presence of PMA, native and recombinant IFN-γ are growth factors for B cells from a B-PLL patient and that IL-2 is not involved in this process.

The superantigen of mitogen- or antigen-activated T cells contains factors with the capacity to regulate B-cell proliferation in vitro. Not only normal human B cells but also malignant B cells, which are considered to be arrested in a particular stage of B cell ontogeny, can be induced to proliferate upon the addition of T cell-derived factor(s).

Two genetically well-defined lymphokines are usually present in such preparations, ie, interleukin 2 (IL-2) and interferon-γ (IFN-γ). Both factors can act on the proliferation of normal and neoplastic B cells. IL-2 stimulates the outgrowth of normal preactivated human B cells. Epstein-Barr virus–transformed cells, and prestimulated B cells from patients with B chronic lymphocytic leukemia (B-CLL). The role of IFN-γ in B cell proliferation is not very well established. IFN-γ does not affect the outgrowth of normal human B cells, whereas in other studies it costimulates proliferation of anti–μ-activated B cells. Unstimulated B-CLL cells have been shown to proliferate in response to IFN-γ. In murine systems, proliferation of B cells is either inhibited or not influenced by IFN-γ, depending on the costimulatory conditions for these cells.

Recently, we showed that B cells from five out of five patients with B prolymphocytic leukemia (B-PLL) proliferated in vitro when incubated with the phorbol ester phorbol myristate acetate (PMA). Upon the addition of T cell–derived conditioned medium, PMA-stimulated B cells of one patient showed a synergistic enhancement in proliferation.

The proliferative effect of PMA on human B cells has been previously described. The aim of the present study was to investigate the nature of the factor in this preparation that was responsible for the increased proliferation. Our data indicate that IFN-γ and not IL-2 is a potent stimulus of B-PLL cell outgrowth and that IFN-γ is at least partially responsible for the proliferation induced by this T cell supernatant.

MATERIALS AND METHODS

Cell Preparations and Separations

B-PLL cells. Isolation of B cells from B-PLL patient W.V. has been described elsewhere. Briefly, mononuclear cells (MNC) from splenic origin were isolated by Percoll density gradient centrifugation (1.078 g/mL) (Pharmacia Fine Chemicals, Uppsala, Sweden). This cell suspension contained 95% B1+, μ−, δ−, γ−, α− cells and 4% T3-positive cells. B-PLL cells were depleted of residual T cells by two cycles of E rosette sedimentation with sheep erythrocytes treated with 2-aminoethylisothiouronium bromide hydrobromide (AET). Nonmalignant MNC, B, and T cells. Tonsil fragments that were obtained from either adults or children undergoing tonsillectomy were minced and passed through stainless steel mesh in Earle’s medium. Mononuclear tonsil cells were obtained by Percoll density gradient centrifugation as described earlier. Non-T cells and T cells were separated by E rosette sedimentation with AET-treated sheep erythrocytes. This procedure was repeated twice. T cells were obtained by sheep erythrocyte lysis with 155 mmol/L buffered NH4Cl for five minutes at 0°C followed by three washing steps. Non-T cells were further enriched for B cells: 107 non-T cells/mL in Earle’s medium were incubated for 30 minutes on ice with a mixture of anti-Leu-1 (0.25 μg/mL), anti-Leu-M3 (1 μg/mL) and anti-Leu-11b (1 μg/mL) (Becton Dickinson, Sunnyvale, CA). Cells were washed and gently rocked in Earle’s medium with 30% (vol/vol) rabbit serum as a source of complement for 45 minutes at 37°C. The cells were washed at least four times before culture and contained more than 95% B1+, μ+ cells and less than 0.5% T3-positive cells.

Production and Partial Purification of Conditioned Medium

Mononuclear tonsil cells (107/mL) and cells from a lymphoblastoid cell line CLB TT (3,000-rad irradiated; 106 cells/mL) as stimulator cells were cocultured in culture flasks (Nunc, Roskilde, Denmark) in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 2% (vol/vol) fetal calf serum (FCS; GIBCO, Grand Island, NY). After 48 hours, the supernatant was harvested, passed over a 0.22-μm Millipore filter (Millipore Corp, Freehold, NJ), and
stored (–20°C) until used. This material will be referred to as MLC-CM. MLC-CM was concentrated 30 times by ultrafiltration over an Amicon YM-10 filter (Amicon Corp, Danvers, MA) and fractionated by gel filtration on an AcA54 column (LKB, Bromma, Sweden) in a buffer with 0.15 mol/L NaCl, 100 g polyethylene glycol (PEG) 4000/mL, and 5 mmol/L HEPES, pH 7.2.

Preparation and Purification of IL-2

Cells (10^6/mL) from the human leukemic T cell line Jurkat were cultured in IMDM in the absence of proteins with 100 µg/mL PEG 4000 and stimulated with phytohemagglutinin (1µg/mL) and PMA (10 ng/mL). After 24 hours the supernatant was harvested, concentrated, and passed over an AcA54 column (LKB) in a buffer with 0.15 mol/L NaCl, 100 µg PEG 4000/mL and 5 mmol/L HEPES, pH 7.2. The IL-2–containing fractions were pooled, concentrated, and applied to a Mono-S column (Pharmacia) in a high-performance liquid chromatography system equilibrated with 50 mmol/L sodium acetate (pH 4.8) and 0.05% (wt/vol) Tween 80. A linear gradient (0% to 100%) was formed with a buffer containing 50 mmol/L sodium acetate, 0.05% (wt/vol) Tween 80, and 500 mmol/ L NaCl. The IL-2 eluted at 270 mmol/L NaCl as a sharp peak. The specific activity of this peak was 2.5 × 10^6 units/A280 nm unit.

IL-2 Assay

IL-2 was measured in a microassay according to Gillis et al. Cells (5 × 10^5 cells/well) of a cloned IL-2–dependent murine cell line (CTLL) were cultured in IMDM supplemented with 5% (vol/vol) FCS, penicillin (100 U/mL), streptomycin (100 µg/mL), and 2-mercaptoethanol (5 × 10^-3 mol/L). After 16 hours, the cells were labeled with 0.2 µCi (7.4 kBq) 3H-thymidine (2 Ci/mmol) and cultured for another four hours before harvesting. The IL-2 concentration was related to a standard IL-2 preparation where 1 unit of IL-2 was defined as the amount of growth factor that caused half-maximal 3H-thymidine incorporation.

Culture Conditions and Reagents

B cells from B-PLL patient W.V. (5 × 10^4 cells/well) were cultured in flat-bottom microtiter plates (Nunc 96F) (0.2 mL) with IMDM supplemented with 5% vol/vol heat-inactivated human pooled serum, 5 × 10^-3 mol/L 2-mercaptoethanol and antibiotics. The cells were harvested after four days after a pulse of 3H-thymidine (0.2 µCi/well = 7.4 kBq/well, specific activity 2 Ci/mmol) and cultured for another four hours before harvesting. The IL-2 concentration was related to a standard IL-2 preparation where 1 unit of IL-2 was defined as the amount of growth factor that caused half-maximal 3H-thymidine incorporation.

Table 1. Induction of Proliferation in B-PLL and Normal B Cells by PMA and/or MLC-CM

<table>
<thead>
<tr>
<th>Additions</th>
<th>B-PLL Cells</th>
<th>Normal B Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Donor 1</td>
<td>Donor 2</td>
</tr>
<tr>
<td></td>
<td>PMI</td>
<td>PMI</td>
</tr>
<tr>
<td>MLC-CM (1:50)</td>
<td>500</td>
<td>5,380</td>
</tr>
<tr>
<td>MLC-CM (1:15)</td>
<td>440</td>
<td>16,150</td>
</tr>
</tbody>
</table>
| MLC-CM (1:5)    | 700         | 17,910         | 490           | 9,000          | 200             | 7,980          

The Effect of Conditioned Medium on the Proliferation of B-PLL Cells and Normal B Cells

When the phorbol ester PMA was added to T cell–depleted B cells from B-PLL patient W.V., a distinct proliferative response was obtained (Table 1). This proliferation increased significantly upon addition of the supernatant (MLC-CM) from normal T cells that were stimulated with allogeneic Epstein-Barr virus (EBV)-transformed cells. In

The combined data indicate that IL-2, either alone or as a

MLC-CM–Induced Enhancement of B-PLL Cell Proliferation Is Independent of IL-2

Previously, IL-2 has been shown to stimulate the proliferation of preactivated normal and malignant B cells. Because MLC-CM contained a small amount of IL-2 (4 U/mL), we investigated whether IL-2 was involved in the proliferative response of the B-PLL cells. Table 2 shows that the addition of a serial dilution of MLC-CM to PMA-stimulated neoplastic B cells resulted in a dose-dependent increase in proliferation whereas a dose of purified IL-2 that by far exceeded the amount present in MLC-CM had no effect. Upon the addition of Mabs against the IL-2 receptor, the MLC-CM–induced proliferation was not affected whereas the response of T cells from healthy individuals was completely blocked. In contrast to PMA-stimulated T cells, no IL-2 receptors were expressed on the B-PLL cell membrane before or after PMA/IL-2 stimulation (not shown). The combined data indicate that IL-2, either alone or as a

Table 2. Role of IL-2 in B-PLL Proliferation to PMA and MLC-CM

<table>
<thead>
<tr>
<th>Cell Source</th>
<th>Added Stimulus</th>
<th>CLB IL-2 R1</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-PLL cells</td>
<td>–</td>
<td>3,820</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>4,800</td>
</tr>
<tr>
<td>MLC-CM, 1:120</td>
<td></td>
<td>8,920</td>
</tr>
<tr>
<td></td>
<td>1:60</td>
<td>11,400</td>
</tr>
<tr>
<td></td>
<td>1:30</td>
<td>13,390</td>
</tr>
<tr>
<td>IL-2, 10 U/mL</td>
<td></td>
<td>14,430</td>
</tr>
<tr>
<td>T cells</td>
<td>–</td>
<td>5,820</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1,150</td>
</tr>
<tr>
<td>IL-2, 10 U/mL</td>
<td></td>
<td>11,900</td>
</tr>
</tbody>
</table>

The presence of PMA, no response was obtained, and the supernatant of unstimulated T cells or EBV-transformed cells had no effect on PMA-induced B-PLL proliferation.

B-PLL cells or purified normal B cells (5 × 10^6 cells/well) were cultured with or without PMA (1 ng/mL) and MLC-CM. Results are given as counts per minute after a four-day culture period.

In the absence of PMA, no response was obtained, and the supernatant of unstimulated T cells or EBV-transformed cells had no effect on PMA-induced B-PLL proliferation. When purified tonsil B cells from two normal individuals were cultured under the same conditions, PMA induced a proliferation comparable to the response obtained with B-PLL cells (Table 1). However, in contrast to the B-PLL cells, MLC-CM did not further enhance the outgrowth of these nonmalignant B cells. These data suggest that mitogen-free MLC-CM contains putative factor(s) that specifically act on the proliferation of PMA-stimulated cells from patient W.V. but not on nonmalignant B cells.
cofactor, is not the active principle in MLC-CM responsible for the observed stimulatory effect on these B-PLL cells.

**Analysis of MLC-CM by Gel Filtration**

After concentration of MLC-CM, the molecular characteristics of the growth factor for these B-PLL cells were analyzed by gel filtration. Figure 1 shows that the growth factor activity eluted in two distinct peaks, which coincided with a molecular mass of approximately 40 and 20 kilodaltons (kD). When the different fractions were tested for the presence of IL-2, one peak of activity eluted together with the second peak of B-PLL growth factor activity at 15 to 20 kD. This finding is in agreement with the previously described molecular weight (MW) for IL-2. Thus, it appears that the cofactor is not identical to IL-2 and acts independently from this lymphokine.

**IFN-γ Induces B-PLL Proliferation in the Presence of PMA and Is the Active Principle in MLC-CM**

In a search for a well-defined lymphokine that could be responsible for the observed B-PLL proliferation, we focused on IFN-γ. The molecular mass of IFN-γ is 20 and 25 kD for the two different glycosylated states of the monomeric form and 40 to 50 kD for the dimeric configuration.13 Considering the data in the previous section, we tested whether IFN-γ obtained by recombinant DNA technology (recIFN-γ) had any activity in this assay system. Figure 2 clearly demonstrates the potent stimulatory effect of this material, which was active only in the presence of PMA. To test whether indeed IFN-γ was the active principle in MLC-CM, we studied the effect of anti-IFN-γ antibodies on the MLC-CM-induced enhancement of B-PLL cell proliferation (Fig 3). Both IFN-γ and MLC-CM responses were blocked by these antibodies, whereas the proliferation of the neoplastic B cells to PMA alone was not affected. Incubation of MLC-CM for 30 minutes at 56°C, which is known to inactivate IFN-γ completely abrogated its effect (data not shown). The combined data strongly suggest that IFN-γ in the MLC-CM preparation is responsible for the increase in proliferation of the PMA-activated B-PLL cells.
IL-2 Is Not a Growth Factor for B-PLL Cells

An indication that IL-2 did not induce proliferation in PMA-stimulated B-PLL cells was obtained by data shown in Table 2. Because the chosen PMA and IL-2 concentrations might have been insufficient, various doses of IL-2 (up to 250 U/mL) were tested in the presence of a titration of PMA. Figure 4 shows that in the presence of PMA, 250 U/mL IL-2 induced a minor increase of B-PLL cell outgrowth compared with that induced by PMA alone. This response was similar to the one with 10 U IL-2/mL (data not shown), which suggests that IL-2 concentrations above 250 U/mL would not lead to a further increase in B-PLL cell proliferation. In contrast IFN-γ at 100 U/mL showed strong B cell growth factor activity. Thus, these B-PLL cells are highly receptive to the growth-promoting activity of IFN-γ whereas IL-2 has virtually no effect.

The Effects of Preincubation With Either PMA or IFN-γ on B-PLL Proliferation

Since both PMA and IFN-γ are able to modulate membrane marker expression in normal and malignant lymphocytes,13,22 we wondered whether preincubation with either PMA or IFN-γ would induce receptors or at least susceptibility to the other stimulus. Table 3 shows that B-PLL cells preincubated with PMA for 24 hours did not respond with proliferation when recultured with IFN-γ alone. In contrast, exposure of these neoplastic B cells to IFN-γ (100 U/mL) induced responsiveness to PMA without additional IFN-γ.

Table 3. Effect of Preincubation with PMA or IFN-γ on B-PLL Proliferation

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>Regent Added</th>
<th>B-PLL Proliferation (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>— —</td>
<td>— —</td>
<td>60 200</td>
</tr>
<tr>
<td>PMA —</td>
<td>— —</td>
<td>2040 6500</td>
</tr>
<tr>
<td>IFN-γ —</td>
<td>— —</td>
<td>80 120</td>
</tr>
<tr>
<td>PMA + IFN-γ</td>
<td>— —</td>
<td>10880 22700</td>
</tr>
<tr>
<td>PMA —</td>
<td>— —</td>
<td>20 110</td>
</tr>
<tr>
<td>IFN-γ —</td>
<td>— —</td>
<td>1140 3070</td>
</tr>
<tr>
<td>PMA + IFN-γ</td>
<td>— —</td>
<td>30 200</td>
</tr>
<tr>
<td>PMA —</td>
<td>— —</td>
<td>5370 12500</td>
</tr>
<tr>
<td>IFN-γ —</td>
<td>— —</td>
<td>20 nt</td>
</tr>
<tr>
<td>PMA —</td>
<td>— —</td>
<td>10380 nt</td>
</tr>
<tr>
<td>IFN-γ —</td>
<td>— —</td>
<td>50 nt</td>
</tr>
<tr>
<td>PMA + IFN-γ</td>
<td>— —</td>
<td>16510 nt</td>
</tr>
</tbody>
</table>

Abbreviation: nt, not tested.

B-PLL cells (10⁵ cells/mL) were incubated with either medium alone, PMA (1 ng/mL) or IFN-γ (100 U/mL) in 1-mL wells. After 24 hours, the cells were harvested, washed (three times), and recultured at 5 x 10⁴ B-PLL cells/well in 0.2 mL with either no stimulus, PMA (1 ng/mL), recIFN-γ (100 U/mL), or both stimuli. The results are given as counts per minute after four days of reculture.

Similar results were obtained after preincubation with 1 U IFN-γ/mL (data not shown). These data suggest that either IFN-γ strongly binds to the B cell membrane and that both IFN-γ and PMA need to be present in the culture well or that IFN-γ induces modulation of these B cells, which subsequently results in an increased capacity of PMA to induce proliferation.

DISCUSSION

In the presence of PMA, several well-defined lymphokines have been shown to regulate proliferation of malignant B cells and lymphoblastoid cell lines in vitro.6,9 In the present study, we have analyzed the factor in T cell-derived conditioned medium that is responsible for the induction of proliferation in PMA-activated neoplastic B cells from a patient with B-PLL. Several lines of evidence indicate that IFN-γ is responsible for the proliferative response: (a) Gel filtration of MLC-CM resulted in two distinct peaks of activity that eluted in the 40- to 50-kD and 20-kD fractions. These MWs are reported for IFN-γ in its dimeric and monomeric form, respectively. (b) Anti-IFN-γ serum blocked the B-PLL proliferation to MLC-CM and PMA but not to PMA alone. (c) In the presence of PMA, recIFN-γ was a potent inducer of B-PLL proliferation. (d) Treatment of MLC-CM at 56°C for 30 minutes, which is known to inactivate IFN-γ,19 abrogated the functional activity of MLC-CM in this system (unpublished results). Neither MLC-CM nor IFN-γ affected the outgrowth of PMA-stimulated or -unstimulated nonmalignant B cells, and this observation is in agreement with previous findings.10 Recently, anti-μ-stimulated nonmalignant B cells were found to proliferate in response to IFN-γ.11,13 In malignant B cells, IFN-γ has been reported to be a growth factor for unstimulated B-CLL cells.13

IL-2 either alone4,9,11 or as an obligatory cofactor23 has
extensively been shown to stimulate the outgrowth of non-malignant and malignant B cells. A small amount of IL-2 was present in MLC-CM, but with the use of anti-IL-2 receptor Mabs and purified IL-2, we showed that IL-2 with or without additional PMA did not influence the proliferation of these B-PLL cells and that IL-2 was not involved in the proliferative capacity of MLC-CM. Additional IL-2 did not increase the stimulatory effect of IFN-γ on the B-PLL cell proliferation. Similar results were recently obtained by Romagnani et al. in nonmalignant B cells.11

Apart from B-PLL W.V. cells used in this study, we tested the functional capacity of cells from four other B-PLL patients to respond to T cell-derived signals.14 Although PMA induced proliferation in all B-PLL, only cells from patient W.V. showed an increased response in the presence of MLC-CM. Three of five B-PLL were able to actively secrete immunoglobulins in vitro, thus illustrating the functional heterogeneity of this type of leukemia, just as previously described for B-PLL.13 This series is of course too small to draw any definite conclusions about the unique proliferative capacity of B-PLL W.V. cells. The relatively high expression of activation marker 4F2 on the cells of patient W.V. (Sauerwein et al., submitted) might be a clue but needs further evaluation.

In the absence of PMA, neither IFN-γ nor MLC-CM were able to induce B-PLL proliferation. PMA has been shown to change the phenotypic marker expression of malignant B cells14,22 and to induce putative B cell growth factor receptors.10 In the same way, PMA could have induced susceptibility to IFN-γ in our system. However, when B-PLL cells were preincubated with PMA (up to a period of 72 hours), subsequently washed and exposed to IFN-γ, no proliferative response was found. In contrast, preincubation with IFN-γ resulted in responsiveness to PMA. IFN-γ is known to induce changes in the market profile of the cell membrane such as an increase in HLA class II expression.19 Although incubation with either PMA or IFN-γ alone induced no particular changes on the membrane of these B-PLL cells, the combination resulted in increased cell volume and HLA class II and 4F2 expression (unpublished results). This suggests that only the stimulating combination of PMA and IFN-γ can push the cells to a higher activation state. IFN-γ might have such a high affinity for these neoplastic B cells that it cannot be simply washed away from the cell membrane after preincubation.

In conclusion, by analyzing the growth-promoting activity of T cell-derived supernatants for neoplastic B cells, we found that IFN-γ was a growth factor for these B-PLL cells in the presence of PMA. This observation should be taken into consideration when IFN-γ is considered as a therapeutic tool for patients with such neoplasias.

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Induction of proliferation of B prolymphocytic leukemia cells by phorbol ester and native or recombinant interferon-gamma

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