Response of Non-T, Non-B Acute Lymphocytic Leukemia Cells to Phorbol Ester

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ABSTRACT

Non-T, Non-B acute lymphocytic leukemia cells were cultured in vitro with or without the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), a potential modulator of differentiation. The eight cases studied were representative of non-T, non-B acute lymphocytic leukemia (ALL) cells and expressed amounts of la antigens varying from $0.9 \times 10^5$ to $7.1 \times 10^6$ molecules/cell; these levels were measured in a cellular radioimmunoassay with 21W4 monoclonal antibody directed at a monomorphic human la determinant. With all cases, TPA caused a significant increase in the level of la. Cultures with TPA expressed 4.3 times the amount of la found on fresh ALL cells, and a correlation was observed ($r = 0.92$) between the level of la following culture with TPA and that found on fresh ALL cells. A 25% increase in the modal volume of ALL cells was also caused by TPA. There was no detectable induction of surface or cytoplasmic immunoglobulin and no change in the expression of the common ALL antigen. Inhibition of [3H]thymidine incorporation and stimulation of 14C-labeled amino acid incorporation were observed in the presence of TPA, suggesting that the increase in la level occurs concurrently with an increase in protein synthesis induced by phorbol ester. Following culture with TPA, a substantial increase in the ability of the ALL cells to stimulate in a mixed-lymphocyte reaction was obtained. These results suggest that ALL cells, like other cell types, are susceptible to the effects of TPA and respond by changes in cell volume, surface antigen expression, and mixed-lymphocyte reaction stimulating capacity.

INTRODUCTION

The majority of leukemic cells from patients with ALL are classified as non-T, non-B ALL because they do not form rosettes with sheep erythrocytes, do not react with monoclonal anti-T cell antibodies, and do not express surface immunoglobulin (11, 26). However, non-T, non-B ALL cells express la antigens and, in most cases, also bear CALLA (4, 13, 27). Several studies have indicated that 20 to 30% of cases referred to as non-T, non-B ALL have a pre-B cell phenotype, since they express cytoplasmic mu-chain (12, 30). B-cell markers identified with monoclonal antibodies and immunoglobulin gene rearrangements in non-T, non-B ALL have suggested that these leukemic cells are committed to the B-lineage (1, 14, 15, 22, 28).

The tumor promoter TPA can be an active modulator of differentiation of both myelogenous and lymphocytic leukemia cells (10, 23, 29). We have demonstrated recently with 10 cases of CLL that, among the changes induced by TPA, there were increases in la expression and in the ability of CLL cells to stimulate in a MLR (24). Such changes were accompanied by the induction of cytoplasmic mu-chain and, in one case studied, by IgM secretion (24, 25). Furthermore, the capacity of the CLL cells to respond to TPA appears to be correlated with their phenotypic markers. CLL cells with low amounts of surface immunoglobulin and relatively low levels of la responded well to TPA, while those cases with bright surface immunoglobulin and elevated amounts of la responded poorly to TPA (24).

In a recent study, we have demonstrated the heterogeneity of non-T, non-B ALL in terms of la expression. In an attempt to determine if the amount of la present on non-T, non-B ALL cells is related to the ability of these cells to respond to TPA, as seen with CLL cells, we measured the levels of la on ALL cells following culture with TPA. We also investigated the effects of TPA on the modal volumes of these cells and on their ability to stimulate in a MLR.

MATERIALS AND METHODS

Patients. Nine patients with newly diagnosed ALL who had not received previous chemotherapy were studied. Their age, sex, and WBC are shown in Table 1. The diagnosis of ALL was based on the morphology of Wright-stained blast cells on bone marrow and peripheral blood smears and on cytological studies including myeloperoxidase, Sudan black B, periodic acid-Schiff, and nonspecific esterase stainings.

Cell Separation, Volume, and Marker Studies. Mononuclear cells from bone marrow or peripheral blood were obtained by Ficoll-Hypaque density gradient centrifugation and washed with PBS, pH 7.25. Cell size distribution was determined using a Model ZBI Coulter Counter linked to a Coulter Channelizer and was calibrated with polystyrene microspheres of 10- and 19-μm diameter (Coulter Electronics, Hialeah, FL). The modal volume corresponding to the peak distribution was calculated and expressed in μm.

Marker studies were performed as described previously (24, 25). The percentage of T-cells was determined by spontaneous rosette formation with untreated sheep erythrocytes and with 2-aminoethylisothiouronium bromide-treated sheep erythrocytes. Surface immunoglobulin and cytoplasmic immunoglobulin were assessed using fluorescent isothiocyanate-conjugated goat anti-human mu-chain (Meloy Laboratories, Springfield, VA). For the determination of cytoplasmic mu-chain, ALL cells were prepared by cytocentrifugation and fixed prior to staining. In the current study, the leukemic cell preparations were essentially free of cells forming rosettes with sheep erythrocytes and of surface immunoglobulin-positive cells; 8 of 9 cell preparations were negative for cytoplasmic mu-chain. Patient K.E. was considered an example of a pre-B cell leukemia, 5J. Okamura, M. Letarte, and E. W. Gelfand. The heterogeneity of non-T, non-B acute lymphocytic leukemia defined by the quantitative expression of la and CALLA antigens, Leukemia Research, in press, 1984.
because smears contained 8 to 10% of cells which stained for cytoplasmic \( \mu \)-chain.

The percentage of la-positive cells was determined by incubation of leukemic cells with monoclonal antibody 21w4 or with the control P3/X63-Ag8 culture supernatant (2, 24). The percentage of CALLA-positive cells was determined using BA-3 monoclonal antibody (18) or control X63-Ag8 culture supernatant (2, 24). The percentage of CALLA-positive plasmic M-chain.

because smears contained 8 to 10% of cells which stained for cyto-
fetal calf serum at 37° in a 5% CO\(_2\) humidified incubator. Because of the

cases were cultured for 1 to 3 days at a concentra-
tion of 1 x 10\(^6\) cells/ml, with or without TPA, in RPMI 1640 medium

Cell Cultures. ALL cells were cultured for 1 to 3 days at a concentra-
tion of 1 x 10\(^6\) cells/ml, with or without TPA, in RPMI 1640 medium supple-
mented with penicillin, streptomycin, and 10% heat-inactivated

Quantitation of la and CALLA Antigens. The amount of la and

Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>WBC (x 10(^9)/liter)</th>
<th>Source</th>
<th>la(^a) (%)</th>
<th>la level(^b)</th>
<th>Volume (fl)</th>
<th>CALLA(^c) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. O.</td>
<td>2</td>
<td>M</td>
<td>7.0</td>
<td>BM(^d)</td>
<td>89</td>
<td>0.9</td>
<td>172</td>
<td>ND</td>
</tr>
<tr>
<td>T. A.</td>
<td>6</td>
<td>M</td>
<td>8.9</td>
<td>BM</td>
<td>81</td>
<td>1.6</td>
<td>152</td>
<td>ND</td>
</tr>
<tr>
<td>M. C.</td>
<td>4</td>
<td>F</td>
<td>1.9</td>
<td>BM</td>
<td>90</td>
<td>2.6</td>
<td>205</td>
<td>ND</td>
</tr>
<tr>
<td>B. R.</td>
<td>7</td>
<td>F</td>
<td>47.0</td>
<td>PB</td>
<td>95</td>
<td>2.7</td>
<td>178</td>
<td>89</td>
</tr>
<tr>
<td>R. O.</td>
<td>5</td>
<td>M</td>
<td>296</td>
<td>PB</td>
<td>95</td>
<td>3.6</td>
<td>138</td>
<td>65</td>
</tr>
<tr>
<td>K. E.</td>
<td>5</td>
<td>M</td>
<td>44.0</td>
<td>BM</td>
<td>97</td>
<td>4.6</td>
<td>193</td>
<td>96</td>
</tr>
<tr>
<td>B. A.</td>
<td>6</td>
<td>F</td>
<td>84.8</td>
<td>PB</td>
<td>77</td>
<td>5.0</td>
<td>210</td>
<td>93</td>
</tr>
<tr>
<td>C. L.</td>
<td>8</td>
<td>M</td>
<td>5.4</td>
<td>BM</td>
<td>98</td>
<td>5.9</td>
<td>160</td>
<td>99</td>
</tr>
<tr>
<td>T. H.</td>
<td>3</td>
<td>M</td>
<td>66.3</td>
<td>BM</td>
<td>99</td>
<td>7.1</td>
<td>195</td>
<td>100</td>
</tr>
</tbody>
</table>

\(^a\) The level of la is expressed in molecules of RAM-Fc x 10\(^5\) bound per cell, estimated under saturating

growth conditions.

\(^b\) BM, bone marrow; PB, peripheral blood; ND, not done.

<table>
<thead>
<tr>
<th>la</th>
<th>CALLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

Chart 1. TPA increasing la levels but not CALLA levels. The amount of la and

MLR. ALL cells cultured with or without TPA were harvested; washed

radioactivity of RAM-Fc ranged from 400 to 700 cpm/ng protein in the

radioactivity present was measured and expressed as cpm

MARCH 1984 1247
cules (2, 9). Thus, we are confident that the cellular radioimmu-

The range observed was from 138 to 210 fl with a mean of 178

1248

CANCER RESEARCH VOL. 44

DR-like) on human cells.

no assay used reflects the quantitative expression of la (HLA-

monoclonal antibody is similar on different HLA-DR-like mole-

polymorphic, and it is likely that the epitope recognized by 21 w4

reacts with the \( \alpha \)-chain of HLA-DR molecules. The latter is not

and 125l-labeled 21w4-lgG, the RAM-Fc/21w4 ratio was shown

in cases where quantitation experiments were done with purified

patients studied here are representative of non-T, non-B ALL.

细胞. This value is not significantly different from that calculated

of RAM-Fc bound/cell with a mean of 3.8 ± 2.0 \( \times 10^5 \) molecules/

The modal volumes of ALL cells are also illustrated in Table 1.

The percentage of cells staining for la did not change significa-

Table 2

Elects of TPA on la expression and volume of ALL cells

la levels were calculated as molecules of RAM-Fc bound/cell under saturating conditions, whereas modal volumes expressed in fl were estimated from Coulter Counter analysis. Ratios were obtained for levels of la or volumes obtained with ALL cells cultured with or without TPA relative to values obtained with fresh ALL cells.

<table>
<thead>
<tr>
<th>Patient</th>
<th>TPA+ cells</th>
<th>TPA- cells</th>
<th>% of la+ cells</th>
<th>Ratios of la levels to fresh ALL</th>
<th>Ratios of volumes to fresh ALL</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. O.</td>
<td>79</td>
<td>86</td>
<td>1.33</td>
<td>5.78</td>
<td>1.09</td>
</tr>
<tr>
<td>T. A.</td>
<td>78</td>
<td>90</td>
<td>0.25</td>
<td>2.44</td>
<td>1.11</td>
</tr>
<tr>
<td>M. C.</td>
<td>84</td>
<td>97</td>
<td>1.84</td>
<td>5.23</td>
<td>1.14</td>
</tr>
<tr>
<td>B. R.</td>
<td>71</td>
<td>91</td>
<td>2.77</td>
<td>5.04</td>
<td>0.96</td>
</tr>
<tr>
<td>R. O.</td>
<td>96</td>
<td>98</td>
<td>3.19</td>
<td>3.58</td>
<td>1.07</td>
</tr>
<tr>
<td>K. E.</td>
<td>99</td>
<td>97</td>
<td>1.89</td>
<td>4.43</td>
<td>1.03</td>
</tr>
<tr>
<td>B. A.</td>
<td>86</td>
<td>91</td>
<td>1.00</td>
<td>4.18</td>
<td>0.88</td>
</tr>
<tr>
<td>C. L.</td>
<td>98</td>
<td>99</td>
<td>3.31</td>
<td>3.81</td>
<td>1.02</td>
</tr>
<tr>
<td>Mean</td>
<td>86 ± 10^6</td>
<td>94 ± 5</td>
<td>1.68 ± 0.96</td>
<td>4.25 ± 1.11</td>
<td>1.01 ± 0.12</td>
</tr>
</tbody>
</table>

Mean ± S.D.
Response of ALL Cells to Phorbol Ester

± 1.11 is observed for TPA+ culture/fresh ALL, whereas a ratio of 1.68 ± 0.96 is obtained for TPA− culture/fresh ALL (Table 2). These ratios are significantly different with a probability value of <0.01. A linear regression analysis was performed between the level of Ia observed on leukemic cells following culture with TPA and that measured on the corresponding fresh ALL cells (Chart 3). A correlation factor of 0.92 was calculated. This analysis suggests that there is, on average, a 4-fold increase in the Ia level induced by TPA in the non-T, non-B ALL cells studied.

Increase in Ia but not in CALLA Expression Induced by TPA. With leukemic cells from 5 patients, the levels of CALLA antigens were measured following culture with and without TPA. Chart 1 illustrates the results obtained with Cases C. L. and K. E. No increase in the level of CALLA was observed in either case, although a significant increase in Ia was induced by TPA. In the 3 other cases studied (data not shown), no changes in CALLA levels were observed while changes in Ia similar to those reported in Chart 2 were seen. Notice that CALLA levels, 1 to 2 x 10^5 molecules/cell (Chart 1), are much lower than Ia levels. In a study of 13 cases assessed simultaneously for Ia and CALLA, an average level of CALLA of 1.1 x 10^5 molecules of RAM-Fc bound/cell (from 0.34 x 10^5 to 2.2 x 10^5) and an average level of Ia of 4.1 x 10^5 molecules of RAM-Fc bound/cell (from 0.6 to 8.7 x 10^5) were estimated.

Chart 1 also illustrates that adding phorbol instead of phorbol ester to the cultures (Chart 1, D) caused a minimum increase in Ia and no change in CALLA. The increase in Ia induced by the phorbol ester TPA is, thus, specific for that compound, since culture with phorbol in medium plus fetal calf serum, or culture with medium plus fetal calf serum alone, caused only a minimum increase in Ia relative to that induced by the tumor promoter.

Effects of TPA on DNA, RNA, and Protein Synthesis. The 2 cases described in Chart 1 were also assessed for their ability to incorporate [3H]thymidine, [3H]uridine, and a mixture of [14C]-valine, threonine, leucine during a 16-hr pulse which followed the 48-hr culture period with or without TPA. Fresh ALL cells were also pulsed for 16 hr. Chart 4 demonstrates that culturing ALL cells from Patient C. L. either in medium plus fetal calf serum alone, or with 1.6 x 10^-9 M phorbol, increased significantly the incorporation of [3H]thymidine. An increase is also observed for ALL cells of Patient K. E. when cultured in medium plus fetal calf serum alone. This increase in DNA synthesis is likely to be due to the presence of mitogenic factors in the fetal calf serum (8). TPA induced a large decrease in [3H]thymidine incorporation in both cases; furthermore, it blocked the ability of fetal calf serum to stimulate DNA synthesis.

Chart 2. Increase in Ia expression of ALL cells cultured in vitro with TPA. All cells were cultured with or without TPA. The amount of Ia present on fresh ALL cells prior to culture (C) was estimated by cellular radioimmunoassay under saturating conditions of monoclonal antibody 21w4 and of RAM-Fc. The Ia level was also estimated following culture with TPA (E) or without TPA (B). Time of culture with TPA was 3 days for GO and TA cases and 2 days for all other cases. Bars, S.D. of Ia saturation levels, expressed in molecules RAM-Fc x 10^5 bound/cell.

Chart 3. Correlation between the level of Ia found on ALL cells cultured with TPA and that of fresh ALL cells. The saturating levels of Ia observed on ALL cells after culture in vitro with 1.6 x 10^-9 M TPA was plotted versus the saturating levels of Ia observed on the same ALL cells prior to culture (data shown in the histograms of Chart 2). Linear regression analysis gives a correlation value of 0.92 between the 2 parameters.
Results observed with [3H]uridine incorporation are also shown in Chart 4. Culturing ALL cells from Patient C. L. in either medium plus fetal calf serum alone, 1.6 × 10^{-9} M phorbol, or 1.6 × 10^{-9} M TPA caused a 2-fold increase in [3H]uridine incorporation. Culturing ALL cells from Patient K. E. with TPA caused a 3-fold increase in [3H]uridine uptake, whereas culturing the cells in medium plus fetal calf serum alone had no effect. It would be necessary to analyze several more cases to verify if TPA consistently causes an increase in RNA synthesis or not.

However, in the 2 cases studied, TPA caused a 3- to 4-fold increase in the incorporation of [14C]labeled amino acids (Chart 4). Culturing the cells with medium plus fetal calf serum alone, or in the presence of phorbol, had no effect on protein synthesis. These results suggest that the increase in la level might be occurring at the same time as an increase in protein synthesis; however, it is likely that the augmentation in the rate of protein synthesis is restricted to certain proteins, as suggested by the observation that the level of CALLA antigen is unchanged in the 5 cases studied.

**Effects of TPA on MLR Stimulatory Capacity of Common ALL.** Following culture in the presence or absence of TPA, ALL cells were tested for their ability to stimulate in a MLR. The values observed in the control group reflect the variation in the capacity of ALL cells to stimulate in the MLR. Cells from Patients G. O., M. C., and B. R. were poor stimulators, whereas cells from Patients T. A., R. O., and T. H. were relatively good stimulators (Table 3). TPA was able to increase by 63- and 38-fold, respectively, the MLR-stimulatory capacity of 2 of the ALL cases classified as poor stimulators, i.e., M. C. and B. R. With G. O., a 7-fold increase in MLR-stimulatory capacity was seen with one responder. TPA was also effective in potentiating the ability of the ALL cells already classified as good stimulators of the MLR. Increase of 2.3, 3.0, and 8.2 were observed for Cases T. A., T. H., and R. O., respectively.

**DISCUSSION**

With the phorbol ester TPA as an active modulator of differentiation (10, 23–25, 29), it was of interest to study its ability to induce changes in non-T, non-B ALL cells arrested at early stages of differentiation. In the present study, we have found, in the 8 cases of non-T, non-B ALL studied, a 4-fold increase in la expression induced by TPA. The mechanism by which TPA causes such an effect is unclear. The increase in the level of la cannot be attributed to an increase in cell size; a 25% increase in cell volume cannot account for a 400% increase in antigen expression at the cell surface. There is a net increase in la density calculated in molecules/unit of surface area, and Chart 4 suggests that the augmentation in the la level might be due to an increase in the amount of la synthesized. In 2 cases studied for amino acid incorporation, a 3- to 4-fold increase in the rate of protein synthesis was demonstrated. However, the latter might be restricted to certain proteins, since no changes in CALLA levels were observed (Chart 1). In mouse epidermis, TPA induced the synthesis of specific proteins in a temporal sequence which reflected the program of differentiation of those cells (5, 16). Our results suggest that TPA induces an increase in la synthesis, although we have not demonstrated it directly by pulse chase experiments and immunoprecipitation. Other factors could be responsible for higher la levels. Unmasking of antigenic sites following treatment with TPA could be secondary to membrane perturbations induced by the drug. Alternatively, conformational changes in la molecules, following TPA treatment, could increase the avidity of the 21w4 antibody for accessible epitopes.

Since the role of la in cells at early stages of lymphocyte differentiation is unknown, it is difficult to assess the functional significance of an increase in the la level. la glycoproteins are implicated in interactions between macrophages and T- and B-cells (3). However, a more general role of la in cellular interactions and/or in regulatory steps involved in differentiation cannot be excluded.

Nadler et al. (21) have recently reported that TPA could induce the expression of B1 antigen and of cytoplasmic μ-chain in several cases of non-T, non-B ALL, but they did not detect any changes in la. LeBien et al. (17) have reported changes in BA-2 antigen in 3 of 7 ALL cases, while no changes were observed in the percentage of cells labeled with BA-1, BA-3, or anti-la antibodies following culture with TPA. If one only considers the percentage of la+ cells, no changes in la can be detected in our study as well; 94 ± 5 and 86 ± 10% of cells were la+ following culture with or without TPA (Table 2). However, when la levels were estimated quantitatively using a cellular radioimmunoassay under saturating conditions, changes in la levels of the order of 4-fold were observed.

We did not detect the production of cytoplasmic μ-chain following culture with TPA. Similarly, LeBien et al. (17) could not induce the production of cytoplasmic μ-chain in a non-T, non-B ALL cell line following culture with TPA for periods of time of up to 96 hr. Cossman et al. (7) have demonstrated recently in one case of ALL the induction of cytoplasmic and surface μ- and κ-
chains following TPA treatment. Both μ- and κ-genes were shown to be rearranged in these leukemic cells prior to TPA treatment, although no cytoplasmic μ-chain production could be detected. The authors concluded that TPA affected the transcription or translation of the effectively recombined genes (17). Thus, it is possible for a non-T, non-B ALL cell to carry functionally rearranged μ- and κ-genes without producing detectable levels of cytoplasmic μ-chain. Since the majority of non-T, non-B ALLs appear to have rearranged immunoglobulin genes and to be genetically committed to the B-lineage, the lack of cytoplasmic μ-chain production following TPA treatment could be explained by nonfunctional μ-chain gene rearrangements in the cases that we studied. In contrast, the leukemias studied by Nadler et al. (21) and by Cossmann et al. (7) would represent cells with efficiently rearranged μ-chain genes which can be triggered by TPA to production of cytoplasmic μ-chain. Alternatively, the fluorescence microscopy detection assay might not be sufficiently sensitive to permit the visualization of very low levels of cytoplasmic μ-chain present in the ALL cases studied here, before and/or after stimulation with TPA.

The ability of non-T, non-B ALL cells to stimulate in an MLR was variable and was not correlated with the level of la present on the cells (Tables 1 and 3). Following incubation with TPA, a substantial increase in la expression was seen in the ability of ALL cells to stimulate in an MLR. We have reported a similar effect of TPA with CLL cells (24). As with CLL cells, the increase in MLR-stimulatory capacity induced by TPA did not correlate with the quantitative increase in la expression. We suggested previously that the effects of TPA on potentiating MLR might be explained by conformational changes in la glycoproteins or by the induction or alteration of determinants other than la which are stimulatory in the MLR (24). Alternatively, the effect on the MLR stimulation by TPA in non-T, non-B ALL is associated with a signal for differentiation in human myeloid cells from patients with acute and chronic myelogenous leukemia. Br. J. Haematol., 47: 203–210, 1981.

It is interesting to speculate that the increase in la induced by TPA in non-T, non-B ALL is associated with a signal for differentiation. Since it is believed that most of these cells are B-cell precursors, it is surprising that TPA can induce the expression in these poorly differentiated cells of la levels reaching those found in mature B-cells and activated T-cells (19). This would imply that a high level of la does not necessarily correlate with a functionally mature B-cell or an activated T-cell but that it can be induced with the proper stimulus in other cells, as seen here, with poorly differentiated non-T, non-B ALL cells.

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