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Cytotoxic effect of dibutyryl cAMP, phorbol-12-myristate-13-acetate and lipopolysaccharide, but not interferon-γ, on promonocytic cell lines *in vitro*

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We studied whether inducers of cell differentiation alone could have cytotoxic effect on the promonocytic U937 and Mono Mac 6 cells *in vitro*. The cells were incubated with standard differentiating doses of interferon (IFN)-γ, dibutyryl cAMP (Bt2cAMP) or the phorbol ester phorbol-12-myristate-13-acetate (PMA), with or without lipopolysaccharide (LPS), and both protein synthesis and viability were examined. In both U937 and Mono Mac 6 cells the incorporation of [^{3}H]leucine was significantly reduced after PMA plus LPS stimulation, but not after IFN-γ stimulation, when compared with controls. For U937 cells there was also reduced incorporation after Bt2cAMP stimulation. Trypan blue exclusion experiments and the number of cells remaining in the cultures indicated that Bt2cAMP-, PMA- and/or LPS-stimulated, but not IFN-γ-stimulated, cells were less viable than unstimulated U937 or Mono Mac 6 cells. The results suggest that Bt2cAMP, PMA and LPS, but not IFN-γ, are cytotoxic towards promonocytic cancer cell lines *in vitro*.

Key words: Cytotoxicity, dibutyryl cAMP, lipopolysaccharide, Mono Mac 6 cells, phorbol-12-myristate-13-acetate, U937 cells.

Introduction

The membrane-permeable cAMP analog dibutyryl cAMP (Bt2cAMP) promotes differentiation of promyelocytic cells,[1,2] and so does the protein kinase C activator phorbol ester phorbol-12-myristate-13-acetate (PMA)[3,4] and bacterial lipopolysaccharides (LPS).[5,6] Interferon (IFN)-γ is an important immunoregulatory cytokine produced by T and NK cells,[7] that among its functions leads to differentiation of monocytic cells and promotion of monocyte effector functions.[8] IFN-γ is shown to induce differentiation of U937 cells and to a lesser degree Mono Mac 6 cells.[9] One main finding when immature cells differentiate into mature cells is that they loose their ability to proliferate and cannot be maintained in culture for as long as their progenitors.

Recently, we found that formyl peptide–toxin conjugates as well as ^{125}I-labeled C5a were capable of killing myeloid cancer cell lines *in vitro*.[10,11] By exploiting the formyl peptide receptor or C5a receptor (CD88) on the cells, either toxins or ^{125}I, respectively, were delivered intracellularly. The cells were pre-stimulated with inducers like IFN-γ,[12] Bt2cAMP[1] and LPS for up-regulating the receptors. Recently, differentiation-inducing agents like dimethylsulfoxide and PMA have been shown to induce apoptosis in U937 and HL-60 cells, respectively.[13,14]

When conducting these cytotoxicity experiments we noted that the incorporation of [^{3}H]leucine in certain stimulated control cells appeared to be lower than in the unstimulated controls and also that the survival rate of the former cells appeared to be diminished. Therefore, the purpose of the present study was to examine whether the survival rate of myeloid cells really decreased after stimulation with inducers of differentiation and whether such stimulators *per se* act cytotoxically towards myeloid cells *in vitro*.

Materials and methods

Cells

U937 cells (ATCC CRL 1593),[13] Mono Mac 6 cells, a gift from Dr Ziegler-Heitbrock,[9] and HL-60 cells (ATCC CCL 240) were cultured in leucine-free RPMI 1640 (Sigma, St Louis, MO) with 10% FCS (Sigma) with/without recombinant IFN-γ (500 U/ml) (Sigma)

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or Bt2cAMP (1 mM) for 2 days, or PMA (5 nM) plus LPS (1 μM/ml, Salmon. MINN 595), or either PMA or LPS for 1 day. Human monocytes were isolated from venous blood of healthy donors according to Bøyum's method[16] and examined on culture day 4 after stimulation with or without PMA plus LPS. The number of cells was counted after Turk's staining.

Examination of the effect of cell inducers on:

[^{3}H]Leucine incorporation. Cells were seeded at 10^5/well in 96-well cell culture plates (Costar, Pleasanton, CA) in leucine-free RPMI and incubated as mentioned above with or without inducers, and then pulsed overnight with 1 μCi/well [^{3}H]leucine (sp. act. 25–50 Ci/mM) (Amersham, Amersham, UK). The cells were of similar age when the experiments were performed. The supernatant was removed, the cells lysed with 1 N NaOH, cellular protein precipitated with trichloroacetic acid (TCA) and radioactivity recorded in a β-counter (LKB, Model 1212) after addition of scintillation fluid. The results are presented as percentage of [^{3}H]leucine incorporation in control cells with medium alone.

Survival. Cell cultures were handled similarly as above, except for omitting [^{3}H]leucine, stained for 10 min at 37°C with Trypan blue, washed and fixed in 2% glutaraldehyde. The percentage of live cells in each cell culture well was determined by Trypan blue exclusion using an inverse phase-contrast microscope.

Statistics

Non-parametric statistics were used throughout: Mann–Whitney *U*-test and Wilcoxon one sample test for examination of differences in one parameter between two independent and dependent groups, respectively. Statistical analysis program was GB-STAT (Dynamics Microsystems, Silver Spring, MD) and $p < 0.05$ was considered significant.

Results

Effect of inducers of cell differentiation on protein synthesis

U937 cells and Mono Mac 6 cells were incubated with standard differentiating concentrations of IFN-γ, Bt2cAMP, PMA plus LPS or LPS alone for 1–3 days, pulsed with [^{3}H]leucine overnight, and the TCA precipitated radioactivity recorded. For both U937 and Mono Mac 6 cells there was no significant difference between the [^{3}H]leucine incorporation in unstimulated and IFN-γ-stimulated cells (Wilcoxon one-sample test $p = 0.28$ and $p = 0.65$, respectively) (Figure 1), but significantly lower incorporation in PMA- plus LPS-stimulated cells (Wilcoxon one sample test, $p = 0.0065$ and $p = 0.0051$, respectively). For U937 cells there was also a significantly lower incorporation of [^{3}H]leucine after Bt2cAMP or LPS stimulation alone compared with the unstimulated control ($p = 0.0051$) (Figure 1). For comparison, [^{3}H]leucine incorporation was also examined in unstimulated or PMA- plus LPS-stimulated monocyte cultures, but the rate of incorporation was very low (c.p.m. values usually in the three figure range, data not shown) and no significant differences were found.

Effect of inducers on survival rate of cells

After incubation of U937 or Mono Mac 6 (Figure 2a and b) cells with the inducers the rate of survival for these cells was determined by Trypan blue dye

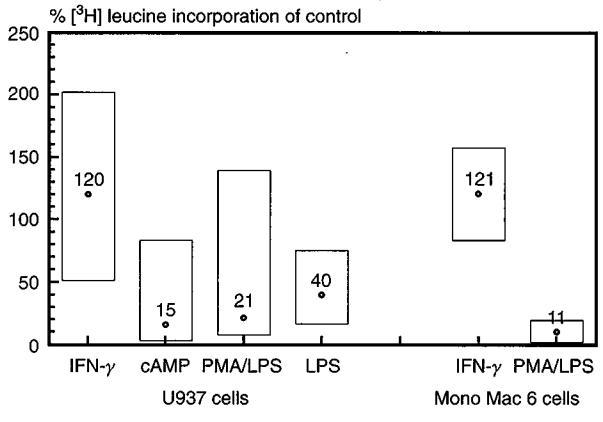

Figure 1. Effect of inducers on cellular protein synthesis. Unstimulated or IFN-γ-, Bt2cAMP-, LPS- or PMA- plus LPS-stimulated U937 or Mono Mac 6 cells were pulsed with [^{3}H]leucine overnight, lysed and TCA precipitated. The radioactivity of the precipitate reflects the [^{3}H]leucine incorporation, which is reported as a percentage of that measured in unstimulated U937 and Mono Mac 6 cells (median: 16 080 and 18 375 c.p.m., range: 7178–59 206 and 15 365–21 385 c.p.m., respectively). The data points for the various U937 and Mono Mac 6 cells above represent medians and ranges of two to nine separate experiments performed in triplicate. Range, □; median, ●.

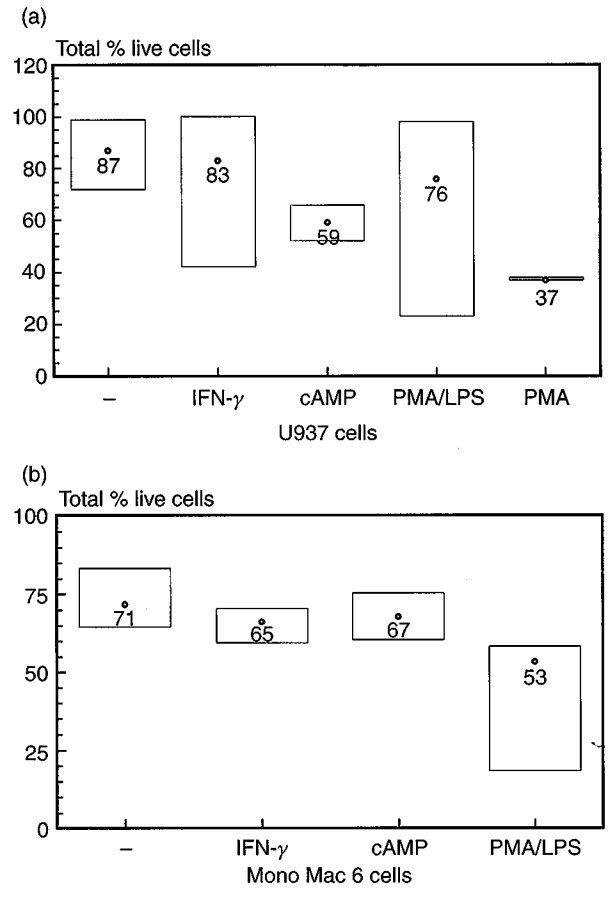

Figure 2. Survival rate of U937 cells (a) and Mono Mac 6 cells (b) treated with inducers. Unstimulated or IFN-γ-, Bt2cAMP-, PMA- or PMA- plus LPS-stimulated cells were stained with Trypan blue, washed and fixed with glutaraldehyde. The percentage of live and unstained cells was scored by microscopy for a cross-section of each cell culture well. The results obtained for the various cells are shown as medians and ranges for two to 12 separate experiments. Range, □; median, ●.

exclusion. In both cell types there was no difference between the rate of survival for unstimulated and IFN-γ stimulated cells (M–W U: $p = 0.60$ and $p = 0.11$, respectively), whereas PMA plus LPS stimulation induced a significantly reduced survival compared with the controls (M–W U: $p = 0.0312$ and $p = 0.0113$, respectively). PMA alone also reduced the number of live U937 cells ($p = 0.0176$). On the other hand, Bt2cAMP stimulation induced a reduction in the survival rate of U937 cells but not Mono Mac 6 cells ($p = 0.0069$ and $p = 0.25$, respectively). The progranulocytic HL-60 cells were also examined in this assay, but neither IFN-γ nor Bt2cAMP stimulation of the cells resulted in significant reduction in the survival rate of these cells (70 and 67%, respectively) compared with unstimulated

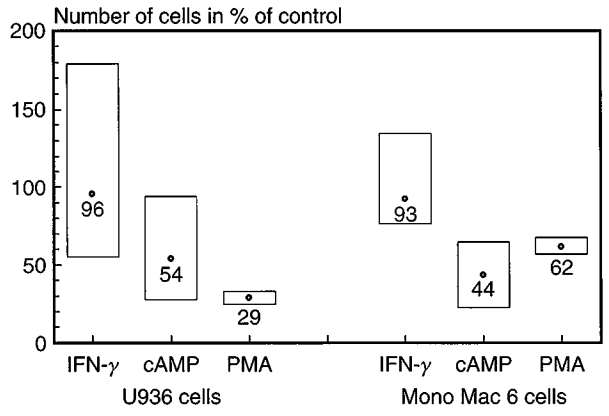

Figure 3. Number of U937 and Mono Mac 6 cells counted in cultures after stimulation with IFN-γ, Bt2cAMP or PMA. Unstimulated cells were aliquoted into new flasks as equal number of cells and in equal volumes and then subjected to inducers during 3 days of incubation. The number of stimulated cells were counted after Turk's staining and data presented as percentage of unstimulated control cells. The results are medians and ranges of two to nine experiments.

HL-60 cells (78%) (M–W U: $p = 0.29$) (not shown). In experiments with PMA plus LPS stimulation of monocyte cultures there was no difference between the survival rate of these (87%) and unstimulated monocytes (88%) (not shown).

Effect of inducers on number of cells in culture

After appropriate stimulation of U937 or Mono Mac 6 cells with IFN-γ, Bt2cAMP or PMA alone, the total number of cells in the cultures were counted. In contrast to IFN-γ (Wilcoxon one sample test: $p = 0.48$ and $p = 0.26$, respectively), addition of Bt2cAMP or PMA to either cell type reduced the total number of cells significantly compared with untreated controls ($p = 0.0077$) (Figure 3). The number of HL-60 cells did not change significantly compared with controls after IFN-γ (85% of control) or Bt2cAMP (102%) stimulation of the cells (not shown).

Discussion

Our *in vitro* results show that PMA and LPS, separate or in combination, as well as Bt2cAMP, reduce both *de novo* protein synthesis and the survival rate in the promonocytic U937 and Mono Mac 6 cells. To our knowledge, we provide the first

evidence for cytotoxicity of Bt2cAMP *per se* towards these cells. In contrast, IFN-γ did not have a cytotoxic effect on the cells. Bt2cAMP did not decrease the survival rate of the myeloid HL-60 cells nor did PMA plus LPS diminish the survival of monocytes.

IFN-γ is used therapeutically mainly for cancer treatment and viral infections *in vivo*, and it inhibits growth of ovarian and breast cancer cell lines *in vitro*.[17,18] Although IFN-γ has been shown to inhibit multiplication and differentiation of myeloid cells, it protects both normal and leukemic myeloid cells from apoptotic cell death.[19] Also, since IFN-γ stimulation of monocytic cells promotes their cytotoxic function towards target cells,[8] our negative finding regarding its cytotoxicity towards the monocytic cells themselves is as expected. Among the interferons, IFN-α is a more likely cytotoxic candidate because it has shown good responses in patients with chronic myelogenous leukemia.[20] However, HL-60 and U937 cells treated with a combination of IFN-γ, tumor necrosis factor (TNF)-α and LPS have diminished viability and cell growth versus controls.[6] Since TNF-α is shown to induce apoptosis in U937 cells,[21] the reported effect[6] was probably caused by TNF-α and possibly also by LPS.

Apart from U937 cells and HL-60 cells being different promyelocytic cell lines, we have no valid explanation for the observed difference between the cytotoxic effect of Bt2cAMP on U937 and HL-60 cells. Bt2cAMP is found to arrest hematopoietic stem cells in G_1 phase without inducing apoptosis.[22] Unfortunately, the effect of LPS and PMA on HL-60 cells was not examined in this study. However, according to the report by Treon and Broitman[6] one would expect a positive cytotoxic effect of LPS, and PMA was previously shown to induce apoptosis in HL-60 cells.[14] Although monocytes may differentiate to macrophages, they are mature cells and the lack of cytotoxic effects from LPS and PMA on monocytes was expected, and so they functioned here as a negative control. However, we observed previously that a concentration of LPS that stimulated human macrophages was toxic for human monocytes.[23] The lesser incorporation of [^{3}H]leucine in Bt2cAMP-stimulated cells and lower numbers of cells in PMA-stimulated U937 than in Mono Mac 6 cells relative to unstimulated controls was probably due to the fact that U937 cells are more immature than Mono Mac 6 cells.[9] In contrast to IL-6, PMA was shown not to induce apoptosis in U937 cells, which indicates that induction of apoptosis is not a consequence of cell differentiation.[24]

The relatively high percentage of live cells observed in the Trypan blue exclusion experiments may be due to an underestimate resulting from disintegration or lysis of dead cells during the long incubation period (48–72 h) with the stimulators and removal during the washing step prior to fixation. This probably also accounts for the discrepancy observed between the survival rate of the Bt2cAMP-stimulated Mono Mac 6 cells determined by Trypan blue exclusion and that found by counting the total cell number. Since the survival rate was lower in experiments with stimulators (except for IFN-γ) than unstimulated controls, one would expect cell loss to be greater for the former cells. Therefore, the difference between survival rates probably is greater than that observed. Since it takes more to kill a cell than to inhibit its metabolism, the effect of the stimulators on viability of the cells was, not unexpectedly, less pronounced than on their protein synthesis.

In view of our present findings, the cytotoxic effect observed previously for formyl peptide–ricin A and –melphalan conjugates and [^{125}I]C5a on Bt2cAMP- and LPS-stimulated U937 cells most certainly is an additive or synergistic effect of the cytotoxicity of the stimulators, conjugates or radiolabeled C5a, respectively.[10,11] The effect of these cytotoxic conjugates and radiolabel were relatively more profound on stimulated than on ustimulated cells in the survival assays of these studies. Therefore, further *in vivo* studies in animal models of monocytic leukemia utilizing such conjugates or radiolabeled peptides should include prestimulation of the animals with inducers of cell differentiation. Provided that [^{125}I]formyl peptide is as cytotoxic towards promonocytic cells as [^{125}I]C5a, [^{125}I]formyl peptide would probably be a better candidate for *in vivo* studies because of the more restrictive distribution of formyl peptide receptors to myeloid cells than C5a receptors (CD88). Another application for inducers of cell differentiation is *ex vivo* stimulation of bone marrow leukocytes from myeloid leukemia patients, with or without addition of conjugates or radiolabel, prior to autologous bone marrow transplantation.

IFN-γ alone does not seem to have cytotoxic effects on myeloid cells, but it may still do so in combination with other inducers.[6] Although LPS showed a cytotoxic effect in the present *in vitro* study of monocytic cells, it is incompatible with treatment of human cancers due to its lethality, e.g. in *Neisseria meningitidis* septicemia. PMA is a non-physiologic agent, but may be applicable *in vivo* in animal studies. Also, Bt2cAMP could be a possible candidate for *in vivo* cytotoxicity studies in animal

models. It is a synthetic second messenger analog to cAMP, and certainly has a range of effects other than cytotoxicity both *in vitro* and *in vivo*. Consequently, both Bt2cAMP and PMA appear worthy of further investigation in animal models of monocytic leukemia.

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