Activation of Cytotoxic Activity of Human Blood Lymphocytes by Tumor-promoting Compounds

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ABSTRACT

Three categories of tumor promoters and chemically related but inactive substances were tested for their effect on the cytotoxic activity of human blood lymphocytes against K562 and Daudi targets. Lymphocytes incubated overnight in the presence of phorbol esters 12-O-tetradecanoylphorbol-13-acetate and phorbol-12,13-dibutyrate [P(Bu)2] had enhanced function. Incubation with 4α-phorbol-12,13-didecanoate was without effect. Enhancing activity was also exerted by the indole alkaloids, teleocidin and lyngbyatoxin A, and the polyacetates, aplysiasatoxin and debromoaplysiasatoxin, but not by dihydroteleocidin. Only the tumor-promoting compounds activated the cytotoxic potential. The substances acted in a dose-dependent manner with optimal activity at characteristic concentrations.

Overnight incubation of lymphocytes at 4°C did not change their spontaneous cytotoxicity but abolished the enhancing effect of P(Bu)2. Thus, P(Bu)2-induced activation occurred only on metabolically active cells. The activation did not require DNA synthesis. Similar to controls, the P(Bu)2-treated cells required divalent cations and an intact cytoskeleton in order to perform their lytic function. Experiments with the various metabolic inhibitors indicate that phorbol ester treatment does not induce an alternative cytotoxic mechanism since, as with untreated lymphocytes, P(Bu)2-activated cells require contact with the target and intact secretory functions.

The enhanced cytolytic potential was not due to induction of α-interferon (IFN-α) production, as shown by the fact that the effect was not abolished by addition of anti-IFN antibodies during the P(Bu)2 treatment of lymphocytes or during the cytotoxic assay. However, the presence of antiserum against IFN reduced the cytotoxic potential of control cells, suggesting that endogenous IFN production contributes to the maintenance of lytic function in cultured cells. If this mechanism is counteracted by addition of anti-IFN serum, the phorbol esters can provide an alternative activation signal. When P(Bu)2-activated lymphocytes were subsequently treated with IFN-α or IFN-γ, their lytic capacity was further increased. These results indicate that P(Bu)2 and IFN activate cytotoxic potential through different pathways.

INTRODUCTION

Phorbol esters and similar diterpenes act as tumor promoters in mouse skin (8, 40). In order to understand the mechanism of tumor promotion, these compounds have been widely studied in vitro systems. They were found to alter the growth behavior, differentiation, and functional characteristics of cells (40). Their effect is initiated by interaction with specific receptors expressed on the plasma membrane (9, 11, 33, 43). These effects were studied extensively with phorbol esters like TPA (8, 40). Indole alkaloids and polyacetates derived from Streptomyces and the blue-green alga Lyngbya majuscula are 2 further classes of tumor promoters chemically unrelated to phorbol esters (10, 13). They interact with the same surface receptor as does TPA and induce similar biological effects (12, 39).

Tumor promoters modify the immune response and the functional behavior of lymphocytes (for review, see Ref. 24). They act as mitogens and enhance the action of other mitogens (15). They stimulate the production of IFN and lymphokines (27, 33, 44, 45). They activate the cytotoxic potential of polymorphonuclear leukocytes (7), and under certain conditions both stimulatory and inhibitory effects on the tumoricidal capacity of TPA-treated macrophages were reported (18, 20, 23).

Phorbol esters modify the cytotoxic activity of lymphocytes. They inhibit the generation of allospecific cytotoxic cells in mixed-lymphocyte cultures but activate the cytotoxic potential of presensitized effectors (2, 26). Depending on the concentration, the length of their interaction, and the presence of regulatory cells, tumor promoters can either enhance or suppress NK activity (for review, see Ref. 19).

We have shown previously that the tumor promoters TPA and P(Bu)2 have a complex modulatory effect on human NK assays because they can influence the behavior of both effector and target cells (30). Long-term incubation with nm concentrations of the compounds resulted in activation of the lytic potential of lymphocytes. This effect was counteracted via activation of the suppressive function of monocytes. The target sensitivity was also modified.

In the present study, we have tested the effect of the indole alkaloids teleocidin and lyngbyatoxin A and the polyacetates apllysiasatoxin and debromoaplysiasatoxin on the human NK system using K562 and Daudi targets. In addition, we analyzed certain characteristics of the P(Bu)2-induced enhancement of the lytic function with special attention to the possible involvement of IFN on the phenomenon.

MATERIALS AND METHODS

Experimental Design. Buffy coat leukocytes were obtained from healthy blood donors. Mononuclear cells were separated by the method of Boyum (4) on Ficoll-Isopaque gradients and were subsequently incubated in a plastic bottle (Falcon) at 37°C in 5% CO2-air for 60 min in order to deplete mononuclear adherent cells. Nonadherent cells were subjected to a nylon wool column chromatography at 37°C in 5% CO2-air for 45 min. The nylon-passed cells were washed twice and counted. Parallel aliquots...
of effector cells were incubated overnight in medium (control) or in the presence of different tumor-promoting agents as described previously (30). The effects of various metabolic inhibitors on the P(Bu)2-induced NK activation were tested. The drugs were added to the cell suspensions 30 min before the addition of P(Bu)2 and left during the incubation period (18 hr) with the phorbol ester. The cells were then washed twice to remove P(Bu)2 and resuspended in a fresh culture medium containing the respective drugs. The lytic potential was measured in a standard 4°C release assay. The viability of the effector cells before the cytotoxic assay was more than 90%.

Medium. RPMI 1640 was supplemented with 10% heat-inactivated newborn calf serum (Gibco Europe U.K.), streptomycin (10 µg/ml), penicillin (120 µg/ml), and glutamine (2 mm).

Reagents. P(Bu)2, 4α-PDD, and TPA were dissolved in DMSO and stored at −20°C (0.2 mm). Teleocidin, lyngbyatoxin A, alypsiatxin, and debromopalyxtoxin (10) were donated by Dr. Richard E. Moore, Department of Chemistry, University of Hawaii. The compounds were dissolved in DMSO and stored at −20°C. The final concentration of DMSO was always less than 0.05%. Na232 (Merck, Darmstadt, Germany), 2-deoxyglucose (Sigma Chemical Co., St. Louis, MO), and 1-β-D-arabinofuranosylcytosine (Sigma) were diluted in medium and used at final concentrations of 10 mM, 50 mM, and 25 µg/ml, respectively. Cytocallasin B (Sigma), colchicine (Sigma), and monensin (gift of Dr. Hans Wigzell, Karolinska Institutet, Stockholm, Sweden) were dissolved in DMSO and used at final concentrations of 20 µM, 10 µM, and 1 µg/ml, respectively. EDTA (KREBO, Stockholm, Sweden) was diluted in H2O and used at 10 mM.

IFN and Anti-IFN Treatment. Parallel aliquots of 10⁶ lymphocytes were incubated for the indicated lengths of time in control medium or in the presence of 30 nM P(Bu)2. To one aliquot, 10⁵ units IFN-α or IFN-γ per ml were added during the last 3 hr before the cytotoxic assay. The cells were washed before their use as effectors [IFN-α (specific activity, 10⁶ units/mg protein) was a gift of Dr. E. Falco, Paris, France]. Sheep anti-IFN-α serum (4.5 × 10⁴ neutralizing units/ml) was a gift of Dr. Kari Cantell, Helsinki, Finland. Different concentrations of the anti-serum were added to the cell suspension 15 min before the addition of P(Bu)2 or IFN.

Cytotoxic Assay. Techniques for the target radiolabeling and cytotoxic assays have been described previously (25, 30). K562 and Daudi cells were washed before their use as targets (4 × 10³) in 20 µl were mixed with 100 µl of triplicated dilutions of effector cells. IFN-γ (specific activity, 5 × 10⁶ units/mg protein) was a gift of Dr. Kari Cantell, Helsinki, Finland. IFN-γ (specific activity, 5 × 10⁶ units/mg protein) was a gift of Dr. E. Falco, Paris, France).

The results are expressed as lytic units/10⁶ cells or percentage of specific ⁵¹Cr release. The number of lytic units from each population was calculated from the dose-response curve and represents the number of killer cells for 15% lysis against K562 and Daudi. The nonpromoter phorbol ester 4α-PDD did not enhance the cytotoxicity.

Pretreatment with the tumor-promoting alkaloids teleocidin and lyngbyatoxin A also activated the cytotoxic function. Dihydroteleocidin, a weak tumor promoter, was not active (10). A nonpromoter phorbol ester 4α-PDD did not enhance the cytotoxicity.

Effect of Metabolic Inhibitors on the Cytotoxic Activity of Lymphocytes

Overnight incubation of the effector cells at 4°C did not affect the subsequently tested lytic activity (Chart 2). However, at low temperatures, the P(Bu)2-induced activation did not occur. Since binding of P(Bu)2 to membrane receptors is shown to occur at 4°C, it is likely that cellular metabolism is required for the P(Bu)2-enhancing effect (9, 33, 43).

Inhibition of DNA synthesis. The presence of 1-β-D-arabino-

### Table 1

<table>
<thead>
<tr>
<th>Target</th>
<th>Phorbol esters</th>
<th>Indole alkaloids</th>
<th>Polycatates</th>
<th>Debranhydratoticin A (50 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4α-PDD (30 µM)</td>
<td>TPA (30 µM)</td>
<td>P(Bu)2 (30 µM)</td>
<td>Dihydrt-cytocallasin (15 ng/ml)</td>
</tr>
<tr>
<td>K562</td>
<td>1</td>
<td>90 ± 10</td>
<td>310 ± 10</td>
<td>166 ± 14S</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>155 ± 5</td>
<td>625 ± 25</td>
<td>250 ± 10</td>
</tr>
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<td>3</td>
<td>108 ± 10</td>
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<td></td>
<td>4</td>
<td>72 ± 8</td>
<td>68 ± 4 (NS)</td>
<td>238 ± 8</td>
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<tr>
<td></td>
<td>5</td>
<td>90 ± 10</td>
<td>96 ± 10</td>
<td>403 ± 27</td>
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<tr>
<td>Daudi</td>
<td>1</td>
<td>14 ± 6</td>
<td>63 ± 7</td>
<td>46 ± 4</td>
</tr>
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<td></td>
<td>2</td>
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<td>49 ± 10</td>
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<td></td>
<td>3</td>
<td>23 ± 1</td>
<td>147 ± 10</td>
<td>138 ± 7</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>16 ± 4</td>
<td>18 ± 2 (NS)</td>
<td>120 ± 10</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>12 ± 2</td>
<td>10 ± 5</td>
<td>312 ± 28</td>
</tr>
</tbody>
</table>

*One lytic unit corresponds to the number of effector cells required for 15% lysis of target.

*Mean ± S.D. of triplicate samples.

cS, significantly different from control (p < 0.01); NS, nonsignificant.
Tumor Promoter Induction of NK Activation

Chart 1. Dose-response curves of the enhancing effect of different tumor promoting agents on the cytotoxic activity of blood lymphocytes. Effector cells were incubated with the compounds for 18 hr. Effector:target ratio, 25:1, Target, K562. Bars, S.D. of triplicate samples.

Chart 2. Effect of temperature on the potentiation of cytotoxicity by PfBu\(_2\). The lymphocytes were incubated for 18 hr in medium (\(\square\)) or in the presence of 30 nm PfBu\(_2\) (\(\bullet\)). Preincubation was performed at 37° or 4°. Target, K562. The results are expressed in lytic units (LU)/10\(^8\) cells. Bars, S.D. of triplicate samples.

Chart 3. Effect of inhibition of DNA synthesis on the potentiation of cytotoxicity by PfBu\(_2\). Control (\(\square\)) and PfBu\(_2\)-treated (\(\bullet\)) cells were incubated in the presence of 1-\(\beta\)-d-arabinofuranosylcytosine (Ara. C). LU, lytic units.

Inhibitors of Cell Respiration and Glucose Metabolism. Inhibition of cytochrome function by 10 mM NaN\(_3\) abolished completely the lytic capacity of control and phorbol-treated effectors (Chart 4). Inhibition of glucose metabolism by 50 mM 2-deoxyglucose gave partial reduction.

Inhibitors of Microtubules, Microfilaments, and the Secretory Apparatus. Complete inhibition of cytotoxicity was obtained by addition of 20 \(\mu\)M cytochalasin B (Chart 5). At this concentration, the drug disrupts microfilament structures (16). Chelating agents also inhibited the lysis. Inhibition of microtubule assembly achieved by 10 \(\mu\)M colchicine reduced the lytic activity of control and PfBu\(_2\)-activated lymphocytes. The addition of monensin, a carboxylic ionophore which prevents release of lysosomal enzymes, also abrogated the cytotoxicity (Table 2) (5, 16).

Role of IFN in PfBu\(_2\)-induced Activation of Lytic Potential

The effector cells were pretreated with PfBu\(_2\) in the presence of anti-IFN-\(\alpha\) serum (Chart 6). When anti-IFN serum was added...
to the control cultures during the 18-hr preincubation, the lytic effect of the cells was reduced, suggesting that maintenance of cytotoxic potential is dependent on activation by endogenous IFN. As expected, in the presence of anti-IFN serum, enhancement of the lytic function by exogenous IFN did not take place. In contrast, the P(Bu)2 activation was not influenced by the antiserum, suggesting that this compound does not act through boosting endogenous IFN production. Moreover, treatment of P(Bu)2-activated cells with IFN concentrations known to induce optimal activation of untreated lymphocytes resulted in a further increase of cytotoxic activity against both K562 and Daudi targets (Table 3). This effect was obtained with both lymphoblastoid IFN (IFN-α) and immune IFN (IFN-γ).

### DISCUSSION

In accordance with previous reports, our results demonstrate that treatment of lymphocytes with the tumor-promoting agents TPA and P(Bu)2 increases their cytotoxic potential under certain conditions (22, 30). The maximal increase occurred after 18 hr incubation in the presence of nw concentrations of the phorbol esters. We tested 2 additional classes of tumor promoters: indole alkaloids, and polycatases such as teleocidin, lyngbyatoxin A, aplysiatoxin, and debromoaplysiatoxin. These compounds share biological activities with phorbol esters and also bind to their plasma membrane receptors (12, 13, 39). Preincubation with low concentrations of the strong tumor promoters, such as TPA, teleocidin, lyngbyatoxin A, and aplysiatoxin, elevated the lytic potential of lymphocytes, while the concentration necessary to achieve similar effects with the weak tumor promoter, debromoaplysiatoxin was 10-fold higher. Incubation with compounds which lack or have much lower tumor-promoting effect, such as 4α-PDD or dihydroteleocidin, had no effect. Thus, we confirmed the findings of Kolb et al. (22), who reported that the 2 effects, tumor-promoting activity and lymphocyte activation, are exerted in parallel.

Phorbol esters were reported to induce opposite effects on the lytic potential of lymphocytes (19). The discrepancies seem mainly to be due to differences in the experimental conditions such as dose, length of treatment, and presence of regulatory cells which can all influence the outcome of the assay. Kolb et al. (22) and Keller et al. (18) reported that preincubation with TPA at low concentrations (10-8 M) results in an enhancement of the NK activity. Goldfarb et al. (14), Abrams et al. (1), and Keller et al. (17-19), using 100 to 1000 times higher concentrations, found that preincubation with TPA suppresses the spontaneous NK activity. Moreover, short-time pretreatment was sufficient to induce the inhibitory effect seen with these TPA concentrations. Seaman et al. (35, 36) reported that mononuclear phagocytes and polymorphonuclear leukocytes are involved in the suppression of NK activity induced by TPA. The mechanism appears to be dependent on the release of reactive forms of molecular oxygen stimulated by tumor promoters.

It is important to notice that TPA is a strong hydrophobic molecule that can bind unspecifically to the lipid component of
the cell membrane (6, 9, 33). It is therefore possible that inhibition of NK activity observed with high TPA doses may be due to nonspecific membrane changes (6, 9). P(Bu)2, the compound that we used for characterization of the effect on cytotoxic activity, is less hydrophobic than TPA. Using P(Bu)2, Driedger et al. (9) and Sando et al. (33) could demonstrate specific phorbol ester receptors on the cell membrane. Saturation of P(Bu)2-specific binding occurred at 30 nM and was reversible in media free of phorbol esters. This dose was found to be optimal for activation of lytic potential (30).

The rapidity of the enhancement of lytic potential suggests that it is not due to alteration of the cell population with subsequent enrichment of certain cell types (22, 30). In accordance, the present experiments show that the DNA synthesis inhibitor, 1-β-D-arabinofuranosylcytosine did not abrogate it. The enhancement of lytic potential induced by P(Bu)2 seems to be an active metabolic process since it occurs only in cells incubated at 37°C and is blocked by inhibitors of cell respiration and glucose metabolism. The information available on the metabolism of TPA indicates that metabolic degradation of the compound is not required for its biological activity (9). Its effects are mediated by binding to specific membrane receptors (9, 12). The metabolic processes activated by phorbol receptor binding are, however, still unknown.

The phenomenon of target lysis can be separated in different steps (32). The first involves establishment of contact between effectors and targets via the plasma membranes. Relevant to the elevation of cytotoxic potential is the finding that phorbol esters and other promoters alter the cell membrane of lymphocytes with conspicuous morphological effect (29). Moreover, the treated lymphocytes aggregate and attach to cells derived from the same species (28, 29). The sialic acid content of the treated cells decreases, which leads to a lower negative surface charge (28, 29, 40). This charge alteration facilitates intercellular contact formation.

Contact between effectors and targets requires divergent cat- ions and intact microfilaments (16, 31, 41). Similar to that in the controls, the cytotoxic effect of P(Bu)2-treated cells was inhibited by EDTA and cytochalasin B. Microtubules are necessary for cytosome organization and cellular secretion, and they were shown to be involved in the mechanism of cell-mediated cytotoxicity (5, 16, 31, 42). Intact secretory apparatus is also necessary for the cytotoxic function of P(Bu)2-treated effector cells as shown by the inhibitory effect of monensin. The enhancement of cytotoxicity may occur through action on secretory organelles since phorbol esters were shown to induce the release of lysosomal enzymes (34).

The experiments with anti-IFN-α serum suggest that the lytic capacity of cultured lymphocytes requires endogenous IFN production. Since the P(Bu)2-treated cells did not change their behavior when they were exposed to this antisera, the phorbol esters do not appear to activate cytotoxicity through induction of IFN production. Our results are therefore in accordance with the finding that IFN was not detected in the supernatant of phorbol ester-treated mouse lymphocytes (22). It remains to be seen whether other lymphokines, such as IL-2, contribute to the enhancement of lytic function. IL-2 was shown to activate lymphocytes for cytotoxicity and to be induced by P(Bu)2 (22, 24, 38, 45).

Combined treatment of lymphocytes with phorbol esters and IFN resulted in additional effects. Both compounds were shown to enhance the lytic potential of low-density lymphocytes, which contain high proportions of NK cells (14, 21, 22, 30). It is still possible that within this subset different cells respond to the 2 stimuli. It is more likely, however, that the same cells are influenced but potentions occur through different pathways. This was suggested by Kolb et al. (22) and Senik et al. (37) on the basis of the need of RNA and protein synthesis for the IFN but not for phorbol ester activation of cytotoxicity.

In conclusion, P(Bu)2 and other tumor promoters can, under certain conditions, increase the cytolytic activity of human blood lymphocytes. The activation does not occur through IFN production. The cytolytic function of activated cells requires contact with the target and an intact secretory mechanism. The experiments suggest that an alternative lytic mechanism does not seem to be generated by phorbol ester treatment. On the other hand, the pathway of activation seems to be different for IFN and phorbol esters.

**REFERENCES**


**Table 3**

Additive effect of IFN and P(Bu)2 pretreatment on the cytotoxic activity of blood lymphocytes

Parallel aliquots (10⁶/ml) of lymphocytes were incubated for 18 hr in control medium, 30 nM P(Bu)2, or IFN (10³ units/ml) alone or in combination.

<table>
<thead>
<tr>
<th>Target</th>
<th>Experiment</th>
<th>Control</th>
<th>P(Bu)2</th>
<th>IFN-α</th>
<th>P(Bu)2 + IFN-α</th>
<th>IFN-γ</th>
<th>P(Bu)2 + IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562</td>
<td>1</td>
<td>120 ± 8</td>
<td>240 ± 5</td>
<td>180 ± 10</td>
<td>312 ± 8</td>
<td>168 ± 7</td>
<td>342 ± 8</td>
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<td></td>
<td>2</td>
<td>94 ± 4</td>
<td>188 ± 8</td>
<td>140 ± 7</td>
<td>220 ± 5</td>
<td>120 ± 7</td>
<td>250 ± 6</td>
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<td></td>
<td>3</td>
<td>297 ± 10</td>
<td>750 ± 15</td>
<td>700 ± 6</td>
<td>840 ± 15</td>
<td>660 ± 16</td>
<td>900 ± 12</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>100 ± 9</td>
<td>300 ± 15</td>
<td>250 ± 12</td>
<td>920 ± 6</td>
<td>450 ± 14</td>
<td>830 ± 8</td>
</tr>
<tr>
<td>Daudi</td>
<td>1</td>
<td>14 ± 5</td>
<td>80 ± 8</td>
<td>76 ± 6</td>
<td>202 ± 4</td>
<td>91 ± 8</td>
<td>150 ± 8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>11 ± 3</td>
<td>65 ± 5</td>
<td>61 ± 3</td>
<td>172 ± 8</td>
<td>86 ± 4</td>
<td>140 ± 9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15 ± 5</td>
<td>85 ± 5</td>
<td>115 ± 7</td>
<td>183 ± 8</td>
<td>90 ± 7</td>
<td>146 ± 4</td>
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<tr>
<td></td>
<td>4</td>
<td>20 ± 6</td>
<td>92 ± 8</td>
<td>150 ± 10</td>
<td>400 ± 15</td>
<td>170 ± 10</td>
<td>450 ± 11</td>
</tr>
</tbody>
</table>

*Mean lytic units ± S.D. of triplicate samples.

**S** significantly different from controls (p < 0.01).

**S** significantly different from P(Bu)2- or IFN-treated samples (p < 0.01).
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