Alteration of Human Lymphokine-activated Killer Cell Activity by Manipulation of Protein Kinase C and Cytosolic Ca2+1

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ABSTRACT

We have examined the effects of protein kinase C (PK-C) stimulation and cytosolic Ca2+ elevation on the in vitro induction of non-histocompatibility-restricted tumoricidal activity from human peripheral blood lymphocytes. The tumor cytolytic activity, as well as the number of cells recovered from interleukin 2 (IL-2)-stimulated cultures, was enhanced by the addition of the PK-C stimulator, phorbol 12,13-dibutyrate (PDBu), but not non-PK-C-activating phorbol ester analogues where the Ca2+ ionophore, ionomycin, did not significantly alter development of IL-2-induced tumor cytolytic activity or enhance cell yield. Neither PDBu nor ionomycin, alone or in combination, induced tumoricidal activity. The addition of both PDBu and ionomycin to recombinant interleukin 2 (rIL-2)-exposed cultures produced a strong mitogenic response and high cell yield, although Daudi cell killing measured at Day 5 was completely abolished. This abrogation of lymphokine-activated killer cell activity was seen as early as 24 h following exposure to PDBu and ionomycin, reaching 50% following 2 days of exposure. When lymphocytes mitogenically expanded by primary exposure to PDBu and ionomycin and then washed free of these agents were further cultured with rIL-2 alone, proliferation continued, and substantial cytolytic activity for Daudi cells was induced. The development of this postexpansion cytotoxic activity was not dependent on the addition of exogenous rIL-2 during the primary cultures. Fractionation of cells into large granular lymphocytes and small T-lymphocytes indicated that only the large granular lymphocytes proliferate in response to rIL-2 alone. Both large granular lymphocytes and small T-lymphocytes proliferate in response to the addition of PDBu and ionomycin, and both populations of cells developed tumor cytolytic activity following removal of PDBu and ionomycin and subsequent culture in rIL-2. These data suggest that PK-C and Ca2+ signals play key roles in the regulation and/or proliferation of tumor cytotoxic lymphocytes or their precursors and that manipulation of those signals can be utilized to produce substantially more tumoricidal activity from lymphocyte populations than can be achieved with rIL-2 alone.

INTRODUCTION

LAK3 cell activity is mediated by a small subpopulation(s) of lymphocytes functionally defined by their ability to kill a variety of solid tumor targets and cultured cell lines by a non-histocompatibility-restricted mechanism following exposure to IL-2 (1). Although many of their immunological activities, including their usefulness for adoptive immunotherapy of cancer (2, 3), have been explored, little is known of the signal transduction mechanisms mediating their growth and functional development.

Phospholipid metabolism is an important mechanism for the transfer of information from ligand-receptor interactions in a wide variety of cell types (4, 5). For T-lymphocytes, substantial evidence indicates that initial antigen-receptor activation signals are mediated by enhanced phospholipid metabolism generating DAG and IP3, which serve to activate PK-C and elevate cytosolic Ca2+, respectively (6, 7). PK-C activation and Ca2+ elevation can trigger several physiological and genetic events culminating in the production and release of lymphokines, such as IL-2, as well as expression of high-affinity receptors for such factors (8, 9). Subsequent interaction between IL-2 receptors and IL-2 induces further physiological events resulting in progression through the cell cycle and development of cell effector functions.

Pharmacological agents which mimic the effects of IP3 and DAG have proven useful as investigative tools for specifically stimulating each arm of this bifurcating signal pathway (4, 5, 10). The ionophore, Io, is highly specific for Ca2+ and can be utilized for initiating extravascular Ca2+ influx into viable cells (11). PK-C activation can be modulated by exposure of cells to PDBu. An important aspect of PDBu is its low lipophihlicity and high receptor dissociation kinetics which allows it to be effectively removed from cells following experimentally defined culture periods (12–14).

Although it is not currently possible to obtain purified populations of LAK cells, since their phenotypic profiles and growth requirements are not yet clearly delineated, pharmacological manipulations of signal transduction events in complex lymphocyte populations may help elucidate mechanisms important for LAK cell regulation and provide insight as to methods for producing large numbers of LAK cells in vitro for more detailed biochemical studies and potential use in adoptive immunotherapy protocols. Since the signals generated from phospholipid metabolism have a critical role in regulating the IL-2 receptor in T-lymphocytes, it is a plausible hypothesis that pharmacological manipulations of PK-C and/or Ca2+ may prove useful in modifying the response of cells mediating LAK activity. Functional enhancement of the LAK cell IL-2 receptor may help alleviate the toxicity associated with high doses of in vivo rIL-2 currently used in adoptive immunotherapy procedures.

In the studies reported here, we examined the effects of PK-C stimulation and cytosolic Ca2+ elevation on the in vitro IL-2 induction of non-histocompatibility-restricted tumoricidal activity from human peripheral blood lymphocytes. Exposure of lymphocyte cultures to PDBu in addition to rIL-2 enhanced the tumoricidal activity of effector cells toward Daudi, an NK-insensitive target. Ionomycin did not alter rIL-2 induction of LAK activity. Tumor cytolytic activity of rIL-2-stimulated lymphocytes was completely abolished by addition of both PDBu and Io. Mitogenic stimulation of lymphocytes with PDBu and Io, followed by removal of the agents and culture with rIL-2...
only, produced substantial tumor cytotoxic activity contributed
to by both LGLs as well as small lymphocytes recovered from
Percoll gradient separation procedures. These data suggest that
PK-C and Ca²⁺ signals play key roles in the regulation and/or
proliferation of LAK cells or their precursors and that manip-
ulation of PK-C and Ca²⁺ can be utilized to produce substan-
tially more tumorcidal activity from lymphocyte populations
than can be achieved by incubation with rIL-2 alone.

MATERIALS AND METHODS

Reagents and Culture Medium. Complete culture medium contained
RPMM 1640 supplemented with 5% human AB serum, 5 mm 4-(2-
hydroxyethyl)-1-piperazineethanesulfonic acid, 50 units/ml of penicil-
in, 50 μg/ml of streptomycin, 2.0 mm glutamine, and 1.0 μM 2-
mercaptopethanol. Tissue culture reagents were obtained from GIBCO
(Grand Island, NY). Experimental agents included human recombinant
IL-2 (Cetus Corporation, Emeryville, CA), ionomycin (Calbiochem,
San Diego, CA), PDBu, and 4o-PDD (L. C. Services Corp., Woburn,
MA). All other chemicals were obtained from Sigma Chemical Co. (St.
Louis, MO).

Isolation of Cells. Lymphocytes were isolated from the venous blood
of healthy volunteers. Mononuclear cells were derived by Histopaque
(Sigma, St. Louis, MO) density gradient centrifugation (15). The cells
were washed via centrifugation, and monocytes were depleted by ad-
herence to plastic tissue culture plates. Following this step, nonadherent
cells were applied to a nylon wool column. The eluted cell population
which was free of monocytes and B-lymphocytes was washed in culture
medium prior to use.

In some experiments, lymphocytes were further separated into small
cells and LGLs using a two-step discontinuous Percoll gradient (16).
The discontinuous Percoll gradients were prepared with layers (top to
bottom) of 43% and 47% Percoll in RPMI 1640 with 10% heat-
inactivated fetal bovine serum adjusted to 285 mOsmol. Lymphocytes
in 10% human AB serum and RPMI were layered on top of the
gradients which were then centrifuged at 400 x g for 20 min at room
temperature. The LGLs were harvested from the 43–47% interface,
while small lymphocytes were recovered from the bottom of the 47%
layer.

Cell Cultures. Cells were cultured in complete medium in T25 flasks
(Costar, Cambridge, MA) with appropriate stimulating agents at 10⁶
cells/ml in 5% CO₂ at 37°C in a humidified incubator. Stimulating
agents were removed by washing the cells via centrifugation 4 times
(400 x g for 10 min) with complete medium at 37°C. The efficacy of
this washout procedure was confirmed by both biological response
analysis as well as by direct measurements of [3H]PDBu (30.8 C/mmol;
New England Nuclear, Boston, MA) retention. Stimulation of lympho-
cytes with either PDBu or Io induces no significant proliferative
response, while costimulation produces very strong proliferation. Treat-
ment of lymphocytes with either PDBu or Io, followed by the afore-
mentioned washing procedure and stimulation with the complementary
agent, produced no mitogenic response. Analysis of [3H]PDBu retention
indicates that lymphocytes exposed to 10⁶ M PDBu retained less than
10⁻⁴ mol of [3H]PDBu/10⁶ cells following washing.

Cytotoxicity Assay. Daudi target cells (10⁶) were labeled with 100
μCi of 5¹Cr (New England Nuclear, Boston, MA) for 2 h at 37°C and
washed extensively prior to use. Targets (5 x 10⁶) and effector cells
were plated in 96-well round-bottomed microtiter plates in 200-μl total
volume of medium at several E:T ratios (80:1, 40:1, 20:1, 10:1). After
a 4-h incubation at 37°C, the supernatants were harvested, and the
radioactivity was determined in a gamma counter. Cytotoxicity was
determined by the amount of ¹¹Cr released from target cells. Three
replicate wells were used for each experimental group, and the percent-
age of specific lysis was calculated as

\[
\text{% of specific lysis} = 100 \times \frac{\text{cpm in experimental wells}}{\text{maximum release}} - \text{spontaneous release}
\]

A LU is defined as the number of effector cells mediating 30% specific
lysis of 5000 target cells. Cytolytic activity was expressed as either
LU per million cells recovered from the culture or as LU per million
precultured cells. The LU per million precultured cells is
calculated based on the number of cells originally placed in culture and
takes into account changes in cell number which may occur during the
incubation period.

\[
\text{LU/10⁶ precultured cells} = \frac{\text{LU/10⁶ recovered cells} \times \text{no. of cells recovered from culture}}{\text{no. of cells originally placed in culture}}
\]

Statistical Analysis. Duncan’s multiple range test was used for deter-
mining statistical differences.

RESULTS

The LAK cell-generating procedure involves the addition of
rIL-2 (100–1000 units/ml) to cultures of human lymphocytes
(1). This procedure induces substantial non-histocompatibility-
restricted cytotoxic activity against several types of freshly
isolated tumor cells as well as the NK-resistant tumor cell line
Daudi. In our system, human peripheral blood mononuclear
cells were depleted of monocytes and B-lymphocytes via plastic
adherence and passage over nylon wool columns. The remaining
lymphocytes were maintained in culture for 5 days with or
without various stimulating agents. At the end of the culture
period, the lymphocytes were extensively washed by centri-
figation and tested for tumor cytolytic activity in a 4-h ¹¹Cr
release assay using Daudi cells as the principal targets. Fresh
allogeneic lymphocytes were used as controls and in no case
were they killed by LAK cell populations (see figure legends).

Table 1 depicts a representative experiment (n = 5) in which
PDBu and ionomycin were tested for their ability to alter the
generation of LAK cell activity in 5-day lymphocyte cultures.
The addition of 1000 units/ml of rIL-2 alone induced substan-
tial cytotoxic activity (17.88 LU/10⁶ precultured lymphocytes),
while unstimulated cultures had very low tumor-killing ability
(0.66 LU/10⁶ precultured lymphocytes). The number of lympho-
cytes recovered at the end of the culture period was generally
70–90% of the number originally placed in culture. Incubation
in medium with or without rIL-2 did not induce measurable
proliferation over a 5-day period as determined from cell counts
(Table 1) as well as [³H]thymidine incorporation (data not
shown). The addition of ionomycin (1.0 μM) alone or with 1000
units/ml of rIL-2 did not induce cellular proliferation in the

Table 1 Alteration of LAK cell activity by PDBu and/or ionomycin

<table>
<thead>
<tr>
<th>Addition</th>
<th>Cell recovery (%)</th>
<th>Lytic units/10⁶ recovered cells</th>
<th>Lytic units/10⁶ precultured cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>74.2</td>
<td>0.93</td>
<td>0.66</td>
</tr>
<tr>
<td>IL-2</td>
<td>89.6</td>
<td>19.91⁺</td>
<td>17.88⁺</td>
</tr>
<tr>
<td>IL-2 + Io</td>
<td>83.3</td>
<td>21.22⁺</td>
<td>17.68⁺</td>
</tr>
<tr>
<td>IL-2 + PDBu</td>
<td>137.5</td>
<td>44.67⁺</td>
<td>61.42⁺</td>
</tr>
<tr>
<td>IL-2 + Io + PDBu</td>
<td>204.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Io + PDBu</td>
<td>175.0</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>Io</td>
<td>61.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PDBu</td>
<td>75.4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Statistically different (P < 0.05) than group with no addition.
⁺ Statistically different (P < 0.05) than group with rIL-2 ± Io.

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cultures. PDBu alone also did not induce proliferation, but did stimulate cell growth when added to cultures along with rIL-2. A very strong mitogenic response, which did not depend on addition of exogenous rIL-2, occurred when both PDBu and Io were added to lymphocyte cultures.

LAK tumoricidal activity was not detected in cultures stimulated with PDBu and/or Io alone. The addition of ionomycin (1.0 μM) to cultures containing 1000 units/ml of rIL-2 did not alter the generation of LAK cells. PDBu (10.0 nM) alone did not induce tumoricidal activity; however, when used in conjunction with rIL-2, LAK activity was significantly (P < 0.05, n = 5) enhanced over that achieved with rIL-2 stimulation alone.

A substantial augmentation of the tumor cytolytic units per million recovered cells was observed when cells were cultured with PDBu and rIL-2 (Table 1). Addition of the non-PK-C-activating phorbol ester, 4αPDD (1 μM), was without biological activity (data not shown). The increase in LU/10⁶ recovered cells, along with enhancement of the total cells obtained from the culture, contributes to the total increase in LU/10⁶ precultured lymphocytes (from 17.88 with rIL-2 alone to 61.42 with rIL-2 + PDBu).

The addition of 10 nM PDBu to lymphocyte cultures enhanced the LAK activity induced over a range of rIL-2 concentrations from 10–1000 units/ml as depicted in Fig. 1. The amount of LAK activity obtained with the PDBu + rIL-2 was greater than could be achieved with rIL-2 alone. When both PDBu and Io were added to lymphocyte cultures, a very strong mitogenic response occurred as measured by an increased number of cells recovered from cultures. However, in cultures costimulated with PDBu and Io along with 1000 units/ml of rIL-2, tumoricidal activity following 5 days of culture was completely abolished (Table 1). To investigate the kinetics of this abrogation of LAK cell activity, 10.0 nM PDBu and 0.5 μM Io were added at various times following rIL-2 stimulation. Depicted in Fig. 2 is the abrogation kinetics of LAK activity following addition of PDBu and Io at various times prior to cytolytic evaluation. Very little inhibition of LAK occurred unless the cells were exposed to PDBu and Io for greater than 24 h prior to cytolytic analysis. The mean time for 50% inhibition of LAK activity was 48 h.

In order to examine whether lymphocytes expanded through stimulation with PDBu and Io could be induced to express LAK cell activity, a series of experiments were performed in which PDBu + Io-expanded cells were washed by centrifugation and then recultured with various agents, including rIL-2.

A comparison of lymphocyte expansion protocols with regard to cellular proliferation and induction of LAK cell activity is depicted in Table 2. Lymphocytes were expanded for 9 days with PDBu and Io with or without a high concentration (1000 units/ml) of human rIL-2. Following the primary stimulation and expansion, the cells were counted, washed, and cultured for an additional 5 days in either medium alone, 1000 units/ml of rIL-2, or the original primary expansion stimuli. At the end of the second culture period the cells were counted, washed, and tested for LAK cell cytolytic activity at several E:T ratios against Daudi targets.

Strong proliferation occurred in the primary cultures resulting in cell expansion from 20 × 10⁶ to 176.8 × 10⁶ for cells stimulated with 10.0 nM PDBu and 1.0 μM Io. The inclusion of 1000 units/ml of rIL-2 in the primary culture resulted in similar proliferation with a cell increase from 20 × 10⁶ to 187.5 × 10⁶. Incubation in complete medium alone during the secondary stimulation results in a loss of viable cells, although some lytic activity was evident in the surviving cells. Exposure of cells to 1000 units/ml of rIL-2 in the secondary culture resulted in a continuation of proliferation of cells expanded by PDBu and Io in the primary culture. The cells expanded by PDBu and Io followed by rIL-2 demonstrated substantial tumor cytolytic activity (8.7 LU/10⁶ recovered cells). This induction of LAK activity in PDBu + Io-expanded lymphocytes was rIL-2 concentration dependent over the range of 10–1000 units/ml (data not shown). Given the amount of cellular proliferation occurring under this regimen, the 127.0 LU/10⁶ lymphocytes originally placed in culture represent substantially more activity, on a per cell basis, than is commonly obtained in standard rIL-2-driven LAK cell generation (10–30 units/10⁶ precultured lymphocytes) (e.g., see Fig. 1) (2, 3). Lymphocytes expanded by PDBu and Io, then maintained in PDBu + Io with or without rIL-2 during the secondary culture period, had very low tumoricidal activity. The inclusion of high concentrations of rIL-2 in the initial PDBu + Io expansion regimen did not significantly alter the later development of LAK activity.

To investigate the proliferative and cytotoxic activity of spe-
Lymphocytes were cultured with stimuli for 10 days during the 1st culture and were fed at Day 5 with fresh complete medium and the appropriate experimental agents. Following the 1st culture, the cells were extensively washed as described in "Materials and Methods" before being cultured an additional 5 days in the 2nd culture. Experimental stimuli included 10 nM PDBu, 500 nM ionomycin, and 1000 units/ml of rIL-2. The cells were washed again following the 2nd culture, and cytotoxicity was measured using Daudi cells as targets. No effector group had greater than 1.0 lytic unit of activity per million recovered cells when normal allogeneic lymphocytes were used as targets. The values are from an individual experiment. Similar results were obtained using cells from five different individuals.

<table>
<thead>
<tr>
<th>Primary culture</th>
<th>% of cell recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDBu + Io</td>
<td>884.0</td>
</tr>
<tr>
<td>PDBu + Io + IL-2</td>
<td>937.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary culture</th>
<th>% of cell recovery</th>
<th>Lytic units/10^6 precultured cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>12.0</td>
<td>PDBu + Io</td>
</tr>
<tr>
<td>PDBu + Io</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>PDBu + Io + IL-2</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>

As indicated by the data in Table 3, LGLs showed a proliferative (i.e., 32.5-fold cell increase) response to rIL-2 during the primary culture phase, while small lymphocytes did not show appreciable proliferation and were of very low viability at the end of the culture period. The LGL population also expressed substantial LAK cell activity (9.58 LU/10^6 recovered cells) following the secondary IL-2-stimulated culture period. Stimulation of cells with PDBu and Io, either with or without exogenous rIL-2, caused substantial proliferation of both LGLs and small lymphocytes.

LAK activity could be induced in both small and large lymphocyte subsets if the PDBu and Io were removed and the cells further cultured with rIL-2. When LAK cell activity was analyzed on a per recovered cell basis, PDBu + Io-expanded LGLs contained 5.45 LU/10^6 recovered cells as compared to 9.58 LU/10^6 recovered cells when rIL-2 was used as a proliferative stimulus in the primary culture. The enhanced growth, however, of LGLs stimulated by PDBu + Io resulted in substantially more lytic units per 10^6 cells originally cultured (1370.0) when compared to cultures receiving only rIL-2 (311.4). These data indicate that the small cell population can be induced to express substantial amounts of LAK activity following stimulation and removal of PDBu and Io and subsequent culture in rIL-2. The data further indicate that, in the LGL population, the amount of LU/10^6 precultured cells can be greatly enhanced by using a similar regime of PDBu + Io to stimulate proliferation prior to induction of tumoricidal activity.

### DISCUSSION

The generation of LAK cell activity is the culmination of complex biological interactions which results in the development of effector cells with non-histocompatibility-restricted tumor cytolytic activity. These cytotoxic cells have shown promise as a treatment for neoplastic disease when adoptively transferred back to the autologous host. Phospholipid metabolism and the generation of Ca^{2+} and PK-C-mediated second messenger signals have been shown to play a key role in transducing signals for a variety of cell functions including cell motility, secretion, and proliferation (4, 5).

While it is not yet clear as to the receptor ligand interactions utilized by cells contributing to LAK activity, it is our hypothesis that phospholipid metabolism and the generation of second messengers which activate PK-C and elevate Ca^{2+} are important transduction events capable of altering the growth and activity of LAK cells. To test this hypothesis, we utilized pharmacological agents which selectively activate PK-C and elevate cytosolic Ca^{2+} levels and determined how these signals alter IL-2 induction of non-histocompatibility-restricted tumoricidal activity in human lymphocyte populations.

Three major conclusions can be drawn from these experiments. (a) The addition of a PK-C-activating phorbol ester, PDBu, to peripheral blood lymphocyte cultures enhanced rIL-2-induced proliferation and growth. (b) The addition of a PK-C-activating phorbol ester, PDBu, to peripheral blood lymphocyte cultures enhanced rIL-2-induced proliferation and growth. (c) The addition of a PK-C-activating phorbol ester, PDBu, to peripheral blood lymphocyte cultures enhanced rIL-2-induced proliferation and growth.

### Table 3: Proliferation and Daudi cytolytic activity of size-fractionated lymphocyte subsets

Human peripheral blood lymphocytes were fractionated into LGLs and small lymphocytes on Percoll gradients. The lymphocytes were cultured 14 days during the primary culture and fed on Days 5 and 10 with fresh complete medium containing the appropriate stimulating agents. The cells were extensively washed as described in "Materials and Methods," then cultured an additional 5 days in the secondary culture. Experimental stimuli included 10.0 nM PDBu, 500 nM Io, and 1000 units/ml of rIL-2. The cells were washed again following the secondary culture, and tumor cytotoxicity was measured using Daudi cells as targets. The values are from a representative experiment. Similar results were observed using cells from three different individuals.

<table>
<thead>
<tr>
<th>Primary culture</th>
<th>Secondary culture</th>
<th>Cell type</th>
<th>Proliferation*</th>
<th>Lytic units/10^6 precultured cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>IL-2</td>
<td>LGL</td>
<td>32.5</td>
<td>9.58</td>
</tr>
<tr>
<td>PDBu + Io</td>
<td>IL-2</td>
<td>Small lymphocytes</td>
<td>0.4</td>
<td>ND*</td>
</tr>
<tr>
<td>PDBu + Io + IL-2</td>
<td>IL-2</td>
<td>LGL</td>
<td>251.2</td>
<td>5.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Small lymphocytes</td>
<td>200.0</td>
<td>1370.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LGL</td>
<td>185.0</td>
<td>4.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Small lymphocytes</td>
<td>180.0</td>
<td>776.8</td>
</tr>
</tbody>
</table>

* Proliferation, number of cells recovered/number of cells at start of culture.

ND, not determined.
2-induced LAK activity, while elevation of cytosolic Ca\(^{2+}\) did not alter LAK activity. (b) The simultaneous stimulation of PK-C and elevation of intracellular Ca\(^{2+}\) abrogated rIL-2-induced LAK activity. (c) Lymphocytes grown by stimulation with PDBu and Io can be induced to express substantial LAK activity following removal of PDBu and Io and further exposure to rIL-2.

Since LAK precursors and effectors are not yet phenotypically well defined and comprise such a small proportion (~1–2%) of blood lymphocyte populations, it is not possible to absolutely determine whether our experimental agents act directly on LAK cells and their precursors or indirectly by triggering events in other cell types which then regulate LAK cell activity. Indeed it appears that several different lymphocyte subpopulations may contribute to LAK cell activity as it is functionally defined (17). Future studies using selective enrichment and depletion techniques of specific lymphocyte subpopulations may address these issues.

The activation of PK-C by the addition of PDBu to IL-2 stimulated lymphocyte cultures increases the LAK cell activity per million recovered cells as well as increasing the number of cells recovered from cultures. In T-lymphocytes, the binding of IL-2 with high affinity receptors triggers proliferation. Protein kinase C has been implicated as having a critical role in the expression of high affinity IL-2 receptors on T-lymphocytes (9). An analogous regulatory event may be involved in mediating LAK activity or the proliferation of LAK precursors, although a definitive proof for such a mechanism awaits procedures for deriving purified populations of the appropriate cells.

Protein kinase C-activating phorbol esters have long been known to modify proliferation and functional behavior of lymphocytes. Depending on the concentration, the length of their incubation, and the presence of regulatory cells, such as monocytes, tumor promoters can either enhance or suppress NK activity (18, 19). In experiments similar to our own in terms of cell populations utilized, PDBu enhanced NK activity, although the role of IL-2 and cytosolic Ca\(^{2+}\) mobilization were not examined (20).

Studies on cloned lines of CTL indicate that PK-C-activating phorbol esters are potent stimulators of the lytic effector process following target cell binding (21). However, pretreatment of CTLs with a PK-C-activating phorbol ester selectively inhibited the lytic reaction distal to the initial target binding event (22, 23). This inhibition is closely correlated with the capacity of various phorbol ester analogues to deplete the CTLs of protein kinase C (24). This study is consistent with the hypothesis that target cell binding by CTLs initiates phospholipid hydrolysis in the CTL and that the subsequent activation of PK-C is important to the lytic mechanism. In contrast to CTLs, long-term incubation of NK or LAK cells with PK-C-activating phorbol esters can augment tumoricidal activity, although potential interactions involving Ca\(^{2+}\) elevation have not been previously addressed. Our data indicate that cytosolic Ca\(^{2+}\) levels play a critical role as to whether lymphocyte cytolytic activity is augmented or abolished by PK-C stimulating agents.

The finding that stimulation of PK-C and mobilization of cytosolic Ca\(^{2+}\) abrogate LAK activity suggests that phospholipid metabolism in lymphocyte populations is important for the regulation of LAK cell activity. This becomes critically important when cell separation procedures, such as positive phenotype selection using receptor-ligand interactions, are used prior to lymphocyte culture. Since many receptor-ligand interactions trigger phospholipid hydrolysis, the stimulation of such signals in lymphocyte populations may alter LAK cell activity. Similar receptor affinity selection procedures, when used just prior to cytotoxic evaluation, however, should not produce alterations of LAK activity mediated by PK-C stimulation and Ca\(^{2+}\) elevation, since this abrogation requires a relatively long period (Fig. 2) to develop. Since PK-C stimulation and Ca\(^{2+}\) elevation cause a secretory response in a variety of cell types, it is plausible that the inhibition of LAK activity may be due to depletion of cytolytic substances from the effector cells. The long time course required for the abrogation of the LAK cell activity, however, would argue against this possibility as those secretory events responsive to PDBu and Ca\(^{2+}\) ionophores in other cell types occur very rapidly.

Stimulation by rIL-2 in the standard in vitro LAK cell-generating procedure does not induce substantial cell proliferation. However, proliferation of lymphocytes in vivo appears to be important for antitumor efficacy of adoptive immunotherapy procedures (3), yet little is known as to how to obtain significant proliferation of LAK cells or their precursors in vitro.

An exciting prospect, in terms of therapeutic potential, is our finding that a substantial increase in LAK activity can be achieved by first growing cells under the influence of PDBu and Io prior to inducing cytolytic function with stimulation by rIL-2. While these initial studies focus only on tumor cytosis in vitro, the in vivo adoptive immunotherapeutic potential of these expanded cell populations is being further explored.

It is very likely that multiple cell types are involved in the regulation as well as effector function associated with LAK cell activity. The LAK effector cell has been distinguished from the NK cell based on phenotypic markers, the IL-2 requirement for induction of cytolytic activity, and the ability to kill tumor targets which are resistant to NK-mediated lysis (2). LAK cell activity has been further distinguished from CTL based on absence of some T-cell phenotypic characteristics (i.e., CD3, T11) in precursor cell populations. Another major difference is the MHC Class I restriction demonstrated by CTLs, whereas LAK cells show no MHC restriction and efficiently kill tumor cell lines which do not express MHC Class I antigens (e.g., Daudi). There is a large body of literature, however, which indicates that CTLs may lose their MHC restriction and begin killing a wide variety of tumor targets (reviewed in Ref. 25). Critical events for the development of such "anomalous killers" include absence of appropriate antigenic stimulation and presence of high levels of IL-2. It is quite possible that the LAK activity seen in our PDBu + Io-expanded lymphocyte populations is due to the activity of such anomalous killers.

Other investigators have identified two cell populations, which can be separated on differential density gradients, which differ in their ability to mediate LAK cytolytic activity (17). Separation procedures using discontinuous Percoll gradients yield LGLs as well as a small, dense cell population consisting primarily of resting (i.e., G<sub>0</sub> phase) T-lymphocytes. The LGL population contains cells mediating NK activity as well as the predominant amount of LAK cell activity following IL-2 stimulation. The small cell population contains no NK activity and very little IL-2-inducible tumor cytotoxic activity (i.e., less than one-tenth of LGLs on a per cell basis).

The small lymphocytes, however, may contain effector cell precursors, but do not develop cytolytic activity in response to IL-2 alone, because they require activation involving signals generated from PK-C stimulation and Ca\(^{2+}\) elevation. Our results indicate the addition of PDBu plus Io stimulates the proliferation of both small lymphocytes as well as LGLs. This proliferation was not dependent on addition of exogenous rIL-2, although there is endogenous production of IL-2 from T-lympho-
cytes of the helper phenotype in these cultures. When PDBu and 10 were washed from the cells, followed by culture in rIL-2, substantial tumor cytotoxic activity was detected in both LGL and small T-cell populations. The small T-cell population thus appears to contain precursors capable of mediating LAK activity once primed by stimulation of PK-C and elevation of cytosolic Ca\(^2\+\). The in vivo antitumor efficacy of this population of cells is the subject of future studies.

The data presented in this study suggest that selective pharmacological manipulation of second messenger systems may contribute to the development of lymphocyte populations with very high antitumor activity. Further analysis of the role played by second messenger systems should provide insights into the biological mechanisms involved in lymphocyte growth and antitumor functional activity. It is hoped that a better understanding of signals affecting LAK cell proliferation and function may greatly improve procedures for the adoptive immunotherapy of cancer.

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Altered of Human Lymphokine-activated Killer Cell Activity by Manipulation of Protein Kinase C and Cytosolic Ca^{2+}

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