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cAMP antagonizes interleukin 2-promoted T-cell cycle progression at a discrete point in early G1

(restriction point)

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ABSTRACT T lymphocytes are stimulated to proliferate in an autocrine/paracrine manner by the lymphokine interleukin 2 (IL-2). In seeking further insight into the mechanisms by which IL-2 induces progression of T cells through the G1 phase of the cell cycle, studies were performed with agents that increase cellular adenosine 3',5'-cyclic monophosphate (cAMP), a well-known inhibitor of lymphocyte growth. The addition of dibutyryl-cAMP, cholera toxin, forskolin, or 3-isobutyl-1-methylxanthine to an IL-2-dependent murine T cell line evoked a dose-related suppression of S-phase transition without affecting cellular viability. Moreover, elevation of cAMP levels led to an accumulation of uniformly small cells, suggesting an arrest in early G1. Consistent with these findings, dibutyryl-cAMP inhibited the incorporation of both [3H]-uridine and [3H]thymidine by IL-2-stimulated, synchronized normal human T cells. Furthermore, maximal inhibition occurred during early G1, as indicated by experiments where the addition of dibutyryl-cAMP was delayed with respect to IL-2 stimulation. Quantitative flow cytometric analysis of RNA and DNA content of IL-2-stimulated cells affirmed that increased cAMP inhibits RNA accumulation and S-phase transition. In addition, exposure of IL-2-dependent, asynchronously proliferating normal human T cells to dibutyryl-cAMP resulted in uniform growth arrest in early G1, the point at which cycling T cells accumulate when they are deprived of IL-2. These results indicate that increased cAMP inhibits G1 progression stimulated by IL-2 and provide a rationale for the use of cAMP analogues as pharmacologic probes for the dissection of cellular events occurring during IL-2 signaling and T-cell G1 transit. They also suggest the possibility of therapeutic immunosuppression by a combination of agents that act at different stages of the T-cell cycle.

Although it is evident that the triggering of IL-2R is responsible for T-cell G1 progression, the molecular mechanisms that actually mediate G1 progression are still obscure. To begin to dissect the events subsequent to IL-2R activation, we have sought pharmacologic antagonists of T-cell proliferation. In this regard, increases in adenosine 3',5'-cyclic monophosphate (cAMP) are known to inhibit the growth of many cell types, including lymphocytes (reviewed in refs. 6–11). Studies with cAMP analogues or agents that elevate cAMP levels have implicated the early events after TCR stimulation as susceptible to suppression (12–14). In particular, increased cAMP inhibits inositolphospholipid turnover, thereby preventing IL-2 production, which normally occurs within a few hours of triggering of TCRs (15–21). However, it has remained uncertain whether all of the inhibition of T-cell proliferation observed after elevation of cAMP levels can be attributed to an effect on TCR-activated IL-2 production, or whether IL-2-mediated G1 progression might also be suppressed. Here we report that a single, early G1 restriction point results from increased cAMP. Consequently, those cellular changes associated with IL-2-stimulated G1 progression do not occur, and the cells remain quiescent, small lymphocytes.

METHODS

Chemicals. N6,O2'-Dibutyryl-cAMP (Bt2cAMP), cholera toxin, and 3-isobutyl-1-methylxanthine were obtained from Sigma. Forskolin was obtained from Calbiochem. Bt2cAMP and cholera toxin were solubilized in RPMI 1640 medium to yield stock concentrations of 20 mM (Bt2cAMP) and 10 µg/ml (cholera toxin). Isobutylmethylxanthine was solubilized in 95% ethanol and forskolin was solubilized in dimethyl sulfoxide at 500 times the final concentrations desired. Freshly prepared stock solutions of all chemicals were diluted to the appropriate final concentration in RPMI 1640 medium just before each assay. All control cultures contained equivalent amounts of vehicle solvent (i.e., ethanol, RPMI, or dimethyl sulfoxide) as the experimental cultures. The addition of ethanol and dimethyl sulfoxide to final concentrations of 0.2% had no adverse effect on cell proliferation.

Homogeneous recombinant IL-2 was provided by Takeda Chemical Industries (Osaka, Japan) as a 1.0-mg/ml solution in ammonium acetate buffer (pH 5.0).

Cell Cultures. Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll/Hypaque discontinuous gradient centrifugation and cultured in complete medium consisting of RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (Sterile Systems, Logan, UT) and 50 units of penicillin, 50 µg of gentamicin, and 200 µg of L-glutamine (GIBCO) per ml. The IL-2-dependent murine cytolytic T-lymphocyte line CTLL-2 was cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, gentamicin, and L-glutamine. One million CTLL-2 cells were cultured with 200 units/ml recombinant IL-2. CTLL-2 cells were deprived of IL-2 by culturing with heat-inactivated recombinant IL-2.

Abbreviations: Bt2cAMP, N6,O2'-dibutyryl-cAMP; IL-2, interleukin 2; IL-2R, IL-2 receptor; TCR, T-cell antigen receptor; PBMC, peripheral blood mononuclear cell.
(subclone 15G) was maintained in Iscove's modification of Dulbecco's modified Eagle's medium as described (22). All cell proliferation assays were performed in triplicate or quadruplicate wells of 96-well plates containing either 10^5 PBMCs or 5 x 10^5 CTLL-2 cells in a total volume of 0.2 ml of medium. Progression through the G1, S phases of the cell cycle was monitored by the incorporation of [3H]thymidine and [3H]thymidine (0.5 μCi per well; 1 μCi = 37 kBq; ICN), respectively. In the experiments evaluating CTLL-2 growth, the cells were cultured with [3H]thymidine for the last 4 hr of a 24-hr incubation period. The percentage of viable cells recovered was determined by the Pronase method (23) as described (24).

**Preparation of IL-2R-Positive, G^+/^- T Cells.** PBMCs were enriched for IL-2R-positive, early G1-synchronized T cells by stimulation of the TCR complex by means of monoclonal antibodies reactive with the T3 (CD3) component of the complex (OKT3, 1:10,000 dilution; Ortho Pharmaceuticals). After a 3-day culture, cells were washed extensively to remove anti-T3 and any IL-2 produced in situ and then were recultured in IL-2-free complete medium for 30–48 hr as indicated. PBMCs cultured in this manner yield a population of early G1-synchronized cells consisting of ~90% T cells, equally represented by the T4+ (CD4+) and T8+ (CD8+) subpopulations (25). Resting cells were then adjusted to 10^6 per ml and 100-μl samples were transferred to microtiter wells. Stimulation of G1 entry and cell-cycle progression of synchronized cells were achieved by the addition of recombinant IL-2 (final concentration, 500 pM); Bt2cAMP was added either simultaneously or after the times indicated.

**Flow Cytometric Analysis of RNA and DNA Content.** Simultaneous measurement of the RNA and DNA content of individual cells was performed by staining with acridine orange (Polysciences, Warrington, PA), followed by flow cytometric analysis (26). Approximately 10^6 cells were stained and analyzed within 90 min on an EPICS V flow cytometer (Coulter). Cells were maintained at 4°C prior to analysis. Fluorescence was generated with a 300-mW, 488-nm argon-ion laser beam. Red fluorescence emission was collected through 488-nm band-pass, 550-nm dichroic, and 610-nm long-pass optical filters. Green fluorescence was collected through 488-nm band-pass, 550-nm short-pass dichroic, and 525-nm band-pass optical filters. Red fluorescence intensity is proportional to the RNA content, whereas the green emission is proportional to the DNA content in each cell (26). The data are based on a sample size of 30,000 cells, were collected through 90° and forward angle light-scatter gates, and were stored by list-mode data acquisition. The percentages of cells in G1 (early G1) and G2/M (late G2) were determined as described (27) with the PARA 1 and quasidlet software on the MDAD computer system (Coulter).

**RESULTS**

**Increased cAMP Inhibits IL-2-Induced T-Cell Proliferation.** To focus directly on the effects of cAMP on IL-2-induced T-cell proliferation, a cloned murine cytolytic T-lymphocyte line (CTLL-2, clone 15G; dependent solely on IL-2 for growth) was used for the initial experiments. IL-2 was used at 10 pM, as this concentration is equivalent to both the equilibrium dissociation constant for IL-2 binding to its high-affinity receptor (Kd) and the concentration required to promote a half-maximal proliferative response (EC50) (1). The membrane-permeant cAMP analogue Bt2cAMP (28) produced a concentration-dependent suppression of S-phase transition, as monitored by the incorporation of [3H]thymidine during the last 4 hr of a 24-hr culture period (Fig. 1A). Similar results were obtained with cholera toxin (Fig. 1B), which increases cellular cAMP by ADP-ribosylation of the stimulatory guanine nucleotide-binding protein (Gs) of the adenylate cyclase system (29). The concentrations effecting 50% inhibition (IC50) were 30 μM (Bt2cAMP) and 0.1 ng/ml (cholera toxin). Forskolin, a direct activator of the catalytic unit of adenylate cyclase (30), and isobutylmethylxanthine, an inhibitor of cyclic nucleotide phosphodiesterase (31), also suppressed IL-2-dependent CTLL-2 proliferation (IC50 = 50 μM and 5 μM, respectively), further indicating that increased cAMP is responsible for the abrogation of growth, rather than a mere nonspecific inhibitory effect by any one agent. The drug-induced suppression was not reversed by increasing the concentration of IL-2, even in the presence of only an IC50 of Bt2cAMP, cholera toxin, or forskolin (data not shown).

The elevation of cAMP levels did not suppress cell proliferation as a consequence of cytotoxicity, as neither Bt2cAMP nor cholera toxin altered cellular viability at any concentration (Fig. 1). However, a discernible morphologic effect did occur after drug exposure: microscopy at the end of the culture period consistently revealed a uniform population of small cells, suggestive of arrest in a resting phase (G0 or early G1) of the cell cycle.

**Increased cAMP Prevents G1 Progression of IL-2-Stimulated, Synchronized Normal T Cells.** Murine CTLL-2 cells maintained in exponential growth with IL-2 represent a highly selected, asynchronously proliferating, cloned cell population. Accordingly, to assess an unselected, synchronized, resting normal T-cell population, human PBMCs were first stimulated by 3 days of culture with anti-T3 to effect expression of IL-2Rs. They were then washed free of any IL-2 produced in situ and allowed to reaccumulate in early G1 during a 2-day culture period in the absence of IL-2. There-
after, a saturating concentration of IL-2 (500 pM) was added and progression through G₁ into the S phase was monitored by [³H]thymidine incorporation. The presence of 250 µM Bt₂cAMP prevented the IL-2-promoted gradual and sustained increase in [³H]thymidine incorporation (Fig. 2A), which is normally followed by a similar increase in [³H]thymidine uptake (Fig. 2B). In contrast, 250 µM sodium butyrate had no effect on IL-2-stimulated cell-cycle progression, indicating that the inhibition by Bt₂cAMP was not due to possible growth-suppressing activity of released butyric acid (32).

From findings such as these it appeared that elevation of cAMP levels suppressed the earliest events for IL-2-promoted G₁ progression. Previous studies had revealed that a critical threshold of IL-2-generated signals accumulates during the first 4–5 hr of IL-2 exposure. Since this threshold must be surpassed before G₁ progression to DNA replication can occur (3), it follows that if elevated cAMP levels block early events of IL-2 activation, then delaying the addition of Bt₂cAMP after stimulation of resting T cells with IL-2 should attenuate the inhibition of cell-cycle transit. Accordingly, to test this assumption, synchronized, IL-2R-positive resting T cells were stimulated by the addition of IL-2 to undergo cell-cycle progression, and Bt₂cAMP was subsequently added at various intervals. The concentration of Bt₂cAMP was chosen to obtain ~75% growth inhibition (IC₅₀, based on Fig. 2). IL-2-mediated G₁ progression then was analyzed by monitoring [³H]thymidine incorporation during the final 2 hr of the 32-hr culture period (Fig. 3). The inhibition of G₁ progression by Bt₂cAMP was quite dependent on the interval after IL-2 stimulation at which the drug was added: maximal inhibition occurred only when Bt₂cAMP was added at the same time as IL-2; the suppression diminished progressively when the addition of Bt₂cAMP was delayed. It is particularly noteworthy that a delay of 24 hr in the addition of Bt₂cAMP abrogated completely its suppressive effect (even when [³H]thymidine incorporation was measured 10 hr later, at 32–34 hr of culture). Identical results were obtained when [³H]thymidine incorporation was monitored. These findings affirm the interpretation that the inhibitory activity of cAMP occurs early in G₁ and obviates nonspecific toxicity as an explanation for its suppressive effects.

Flow Cytometric Analysis Confirms an Early G₁ Block by Bt₂cAMP. Although these studies consistently pointed to early G₁ as the cell-cycle stage likely influenced by cAMP, this interpretation necessarily depended upon results obtained with the total cell population studied at intervals during G₁. More convincing data could be provided by flow cytometric analyses, as they would permit a quantitative assessment of RNA and DNA content of individual cells and thereby allow the determination of the actual proportion of cells moving through G₁ to the later phases of the cell cycle. Accordingly, acridine orange staining followed by flow cytometric analysis was carried out to evaluate changes in RNA content (red fluorescence) and DNA content (green fluorescence) (26) at intervals after the addition of Bt₂cAMP [500 µM (IC₅₀)] to early G₁-synchronized, IL-2-stimulated normal human T cells. Data representative of three separate experiments are displayed in Fig. 4 and Table 1. Control cells, activated by anti-T3 for 3 days and then washed and cultured without IL-2 for 2 days, reaccumulated almost totally in the early G₁ (G₁,α) phase of the cell cycle (Fig. 4A; Table 1). In contrast, aliquots of these cells reexposed to IL-2 resumed cell-cycle progression within 24 hr, a change that was even more prominent after 40 hr of IL-2 exposure (Fig. 4B and Table 1). On the other hand, as anticipated from the results obtained by monitoring radiolabeled uridine and thymidine incorporation, Bt₂cAMP prevented the IL-2-mediated progression through G₁, resulting in a cell population with a fluorescence profile and cell-cycle distribution essentially identical to that of the resting control cells (Fig. 4C and Table 1). In the absence of IL-2, Bt₂cAMP had no additional effect on the distribution of the cells in the various stages of the cell cycle (Table 1).

To complement the data obtained with synchronized cells, asynchronously proliferating IL-2-dependent normal human...
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**Fig. 4.** Flow cytometric analysis of RNA and DNA content of IL-2- and Bt2cAMP-treated PBMCs. Acridine orange staining and dual-parameter analysis for RNA content (red fluorescence) and DNA content (green fluorescence) was performed with a Coulter EPICS V flow cytometer. (A) Resting, early G1-synchronized T cells obtained by IL-2 deprivation as described for Figs. 2 and 3. (B) Resting T cells stimulated with 500 pM IL-2 for 24 hr. (C) Resting T cells treated with IL-2 and Bt2cAMP (25 μM) for 24 hr. (D) Asynchronously proliferating T cells obtained by growth of PBMCs with anti-T3 for 3 days followed by supplementation with recombinant IL-2 for 2 days. (E) Continually cycling T cells prepared as in D except for the addition of Bt2cAMP (500 μM) during the last 30 hr of culture. Results are representative of three separate experiments.

T cells were examined to define whether single or multiple cAMP-inhibition points (restriction points) exist during cell-cycle transition. Based on our observation that IL-2-dependent CTLL-2 cells become morphologically uniform and small after exposure to Bt2cAMP or chelera toxin, we anticipated a discrete growth arrest in early G1. Accordingly, PBMCs were stimulated with anti-T3 for 3 days, at which time 500 pM IL-2 was added for 2 days more. The effect of Bt2cAMP (500 μM) was then monitored after an additional 30 hr of culture, as this time interval represents the approximate mean time for T cells to complete a single growth cycle. The results (Fig. 4 D and E; Table 2) reveal that addition of Bt2cAMP results in a concentration-dependent accumulation of cells in early G1, without arrest at any other phase of the cell cycle. Consequently, the stage at which increased cAMP arrests growth is identical to that occurring after deprivation of IL-2 (compare Fig. 4 A and E; Table 2).

**DISCUSSION**

All of the results support the conclusion that elevation of cAMP levels antagonizes IL-2-stimulated T-cell G1 progression, leading to the accumulation of asynchronously proliferating cells at a very early stage of G1. It bears emphasis that Bt2cAMP, chelera toxin, forskolin, and isobutylmethylxanthine all produce similar results, even though these agents increase cAMP by different mechanisms. Moreover, sodium butyrate itself is without effect, and none of the agents used evoke nonspecific cytotoxicity. Accordingly, the phase at which increased cAMP blocks T-cell proliferation appears to be the same locus of the T-cell cycle at which IL-2 acts.

This finding is reminiscent of previous work with murine embryonic 3T3 cells (fibroblasts): a single G1 restriction point in the 3T3 cell cycle is produced by two diverse situations—i.e., either serum deprivation or Bt2cAMP administration (33). Consequently, the observations made with T cells and those made with 3T3 cells suggest that elevation of cAMP levels ultimately results in the lack of accumulation, or the inhibition, of factors critical for G1 progression. In this regard, it was only possible to examine the effect of increased cAMP on T-cell growth after a detailed series of experiments had finally established that IL-2 stimulates T-cell G1 progression, rather than activation of the TCR as assumed formerly (1-5). Like the 3T3 cell cycle (34), the earliest phases of the T-cell cycle can now be divided into the distinct stages of "competence" and "progression" (3). Thus, it is evident that TCR activation results in T-cell competence in a manner closely resembling the acquisition of 3T3 cell competence stimulated by platelet-derived growth factor and that IL-2 stimulates G1 progression.

It is intriguing that increased cAMP antagonizes both the TCR complex (15-21) and the IL-2R, since the immediate biochemical pathways activated and the biologic effects stimulated by these two receptor systems differ. The TCR operates through the hydrolysis of phosphatidylinositol and results in the transcriptional activation of specific genes including c-fos, c-myc, and genes encoding lymphokines such as IL-2 and the IL-2R (reviewed in ref. 35). By comparison, the immediate biochemical events following IL-2R triggering are unknown and do not appear to involve phosphatidylinositol turnover (36). Further, the genes under IL-2 control seem to be distinct from those under TCR control and have only recently begun to be identified (5). However, if a common mechanism can be found to be responsible for the cAMP inhibition of both of these receptor systems, the information gained could contribute to our understanding of the molecular reactants in the respective activation pathways.

**Table 1. Cell-cycle analysis of IL-2-stimulated resting T cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Duration, hr</th>
<th>Percentage of cells</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>G1A</td>
<td>G1B</td>
<td>S</td>
<td>G2 + M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>24</td>
<td>78</td>
<td>19</td>
<td>9</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>IL-2 + Bt2cAMP</td>
<td>24</td>
<td>77</td>
<td>20</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bt2cAMP</td>
<td>24</td>
<td>80</td>
<td>19</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>40</td>
<td>35</td>
<td>36</td>
<td>20</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2 + Bt2cAMP</td>
<td>40</td>
<td>79</td>
<td>18</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bt2cAMP</td>
<td>40</td>
<td>79</td>
<td>20</td>
<td>1</td>
<td>1</td>
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<td></td>
</tr>
</tbody>
</table>

Resting cells were obtained by 30-32 hr of IL-2 deprivation of cultures activated with anti-T3 for 80-84 hr. Control cells were not treated further. For treated cultures, recombinant IL-2 was added at a final concentration of 500 pM and Bt2cAMP was added at a final concentration of 500 μM. Results represent one of three similar experiments.

**Table 2. Cell-cycle analysis of Bt2cAMP-treated proliferating T cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of cells</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>G1A</td>
<td>G1B</td>
<td>S</td>
<td>G2 + M</td>
<td></td>
</tr>
<tr>
<td>IL-2 washout</td>
<td>34</td>
<td>34</td>
<td>24</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>25 μM Bt2cAMP</td>
<td>38</td>
<td>33</td>
<td>21</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>500 μM Bt2cAMP</td>
<td>65</td>
<td>23</td>
<td>9</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>500 μM Bt2cAMP - IL-2</td>
<td>72</td>
<td>23</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Results represent two experiments. Control PBMCs were cultured for 3 days with anti-T3 and then maintained in 500 pM IL-2 for the remainder of the experiment (30 hr). For IL-2 washout, PBMCs were cultured as above except that IL-2 was washed out before the last 30 hr of culture. For Bt2cAMP treatment, PBMCs were cultured under control conditions (maintained in 500 pM IL-2) except for the addition of Bt2cAMP (25 or 500 μM) for the last 30 hr or were cultured as "IL-2 washout" except for the addition of Bt2cAMP (500 μM) for the last 30 hr.
By comparison, IL-2 is the only G1-progression stimulus for T cells, and no additional nutrients or hormonal components supplied by serum are necessary for IL-2-mediated T-cell G1 progression (41). Therefore, it is of interest that cAMP blocks T cells at the earliest stage of G1, precisely the point at which IL-2 acts. Whether this difference in the localization of the cAMP effect on G1 in 3T3 cells and T cells is important must await information regarding the structure of the p75 α chain of the IL-2R, since this chain is responsible for IL-2 signal transduction (42).

Whatever the nature of the signals triggered by IL-2, the results of the present experiments indicate that they may never even be initiated when cAMP is increased. Since the effects of cAMP are mediated by cAMP-dependent protein kinase (43–47), a phosphorylation event may alter signal transduction by the IL-2R. This could occur as a consequence of phosphorylation of the IL-2R itself or of the molecules activated by the receptor or, alternatively, as the result of a regulatory event affecting the expression of the genes that are specifically activated by IL-2. For example, the interaction of IL-2 with its receptor stimulates the expression of transferrin receptors (48), which facilitate the cellular uptake of iron, a cofactor necessary for optimal synthesis of nucleotides necessary for DNA replication (49). cAMP has been reported to inhibit expression of transferrin receptors (16), a result that is now understandable, given that increased cAMP blocks IL-2 stimulation at a very early stage, well before transferrin receptors are expressed.

Regardless of the precise mechanisms whereby cAMP produces its effects, the present observations indicate that it is possible to antagonize IL-2-promoted T-cell immune responses by using agents that increase cAMP. By comparison, the two immunosuppressive drugs most commonly used, glucocorticoids and cyclosporine, function exclusively by inhibiting the production of IL-2 (5). Inasmuch as increased cAMP blocks both IL-2 production and IL-2 activity, it should be possible to potentiate the effects of glucocorticoids and cyclosporine by adding cAMP-active agents to the present immunosuppressive regimens. It should likewise be feasible to decrease the dose of the potentially toxic glucocorticoids and cyclosporine by using them in conjunction with agents that increase cAMP. In this regard, it is noteworthy that chelera toxin and prostaglandins E1 and E2, agents known to elevate levels of cAMP, have already been shown to have effective immunosuppressants (50–52).