REGULATORY SUBSTANCES PRODUCED BY LYMPHOCYTES

VI. Cell Cycle Specificity

of Inhibitor of DNA Synthesis Action in L Cells*

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Inhibitor of DNA synthesis (IDS)[1] is a glycoprotein of approximately 80,000 mol wt and isoelectric point 2.5–3.0, produced in vitro by concanavalin A (Con A)-activated normal rat lymphocytes or by lymphocytes from a donor immunized with antigen in vivo subsequently rechallenged with the same antigen in vitro (1, 2). We previously demonstrated that IDS completely inhibits phytohemagglutinin-induced DNA synthesis of rat lymphocytes and the proliferation of a mouse fibroblast cell line (L cells; 1). In both cases the effect was not due to cytotoxicity and was completely or partially reversible. IDS is synthesized by lymphocyte subpopulations demonstrating suppressor cell activity and is seen in large amounts in situations where nonspecific suppressor cell activity is enhanced, such as after intravenous injection of large tolerogenic doses of protein antigens (3). Thus, IDS may play a significant role in the nonspecific suppression of immune responses associated with prolonged high levels of antigenic stimulation, as in competition of antigens (4), parasitic infections (5), or tumors (6).

In analyzing the mechanism of action of IDS, we were struck by the fact that in lymphocytes, IDS is inhibitory only when present during a discrete time interval during the mitogen response, namely, between 16 and 24 h after adding mitogen (1). To see whether this finding could be related to a general cell cycle specificity of IDS action, we undertook the studies described below using L cells synchronized by various methods. During the course of these studies, evidence was obtained showing that IDS activated adenylate cyclase and raised intracellular cAMP levels of lymphocytes (7); consequently, the role of cAMP in IDS action was also investigated.

The term "chalone" is often used for inhibitory substances produced by various tissues that control the growth rate of those same tissues, all of which show cell cycle specificity and some of which have been shown to act by altering cAMP/cGMP levels (8). IDS may, therefore, be included in this group as a lymphocytic chalone. It would, however, be the first such substance that does not demonstrate strict tissue specificity because it also works on fibroblasts. The fact that IDS produced by lymphocytes is also active on fibroblasts is one of many examples in which cells such as fibroblasts have been shown to produce or respond to factors usually associated only with lymphocytes. The most obvious, and somewhat trivial example, is lymphotoxin, a factor produced by

[1] *Abbreviations used in this paper:* Con A, concanavalin A; FCS, fetal calf serum; IBMX, isobutylmethylxanthine; IDS, inhibitor of DNA synthesis; PGE_1, prostaglandin E_1, TCA, trichloroacetic acid.

lymphocytes that kills a variety of target cells (9). In addition, fibroblasts and other nonlymphoid cells can be stimulated to proliferate by macrophages (10) and under appropriate conditions can make migration inhibitory factor (11) and nonspecific helper cell factors for an antibody response (12). These findings may reflect an as yet poorly defined interaction between these cell types in controlling inflammation or, alternatively, may represent a common ancestry of the two cell types or the adaptation and refinement by the lymphocytes of a primitive factor associated with cell recognition or growth control. Cultured fibroblast cell lines, unlike lymphocytes, offer a homogeneous population of cells that are long-lived in vitro and readily synchronized, making them ideal targets to study cell cycle phenomena.

Materials and Methods

Production of IDS.   IDS was produced in the supernate of Con A-stimulated rat thymocytes or lymph node cells as described earlier (1, 3). Briefly, the cells were cultured at a density of 5 × 10^6 cells/ml in 50 ml RPMI supplemented with 10% fetal calf serum (FCS) and 2 μg/ml Con A for 48 h at 37°C. The cells were then washed and recultured in serum-free and mitogen-free RPMI for an additional 48–72 h. The supernate was collected at 24-h intervals, concentrated on an Amicon Diaflo apparatus (Amicon Corp., Lexington, Mass.) with a UM-20 membrane, and fractionated on Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N.J.). Active fractions were further purified by preparative isoelectric focusing for 48 h at 1,000 V on an LKB-8100 column (LKB Instruments, Inc., Rockville, Md.) in 1% ampholine solution with a pH range of 2.5–4.0, dialyzed against large volumes of RPMI for 48 h, and assayed on L cells (see below) in a final concentration of 25% in each microwell. In most cases the supernate was concentrated 10-fold, representing the lowest concentration of IDS that when tested in a 1:4 final dilution in the microwell inhibited I cell or Con A-stimulated lymphocyte DNA synthesis more than 90%; and this concentration was used in all experiments. Control fluids, prepared and purified the same way except with mitogen added only at the end of culture, showed little or no inhibitory activity.

Assay of IDS in L Cells.   The A-9 subline of mouse L cells (13; kindly donated by Dr. Nancy H. Ruddle, Department of Epidemiology and Public Health, Yale University School of Medicine) was maintained as a monolayer culture in plastic flasks in Eagle's minimum essential medium supplemented with antibiotics and 10% FCS. To test IDS, cells from a flask of exponentially growing cells were treated with Viokase (VioBin Corp., Monticello, Ill.), washed, counted, and resuspended to 2.5 × 10^6 cells/ml and 0.02 ml (5 × 10^4 cells) added to individual microtiter wells along with a final concentration of 10% FCS plus the supernatant fraction being tested. The total volume was brought to 0.08 ml with RPMI, and the cells were incubated for the desired time at 37°C. DNA synthesis was assayed by adding 1 μCi of tritiated thymidine (spec act 2 Ci/mmol) during the final 24 h and counting the trichloroacetic acid (TCA)-precipitable radioactivity (1). In some experiments, inhibition of L-cell proliferation by IDS was assayed by mitotic index. Cells were arrested in metaphase by adding 1 μg/ml colcemid, usually for 4 h, and the percentage of cells in mitosis counted on aceto-orcein-stained slides.

Synchronization of L Cells.   L cells were synchronized by one of three methods. (a) Mitotic shake off: This method is based on the fact that L cells during mitosis round up and detach from the substrate, and thus relatively pure populations of mitotic cells can be collected without perturbing the population (14, 15). L cells were grown in glass 16-oz prescription bottles in calcium-free medium (16) until nearly confluent monolayers were obtained. After washing the monolayer gently to remove dead or floating clumps of cells, the growth medium was collected every 15 min for several hours, the collected cells being kept in an ice bath in the interim. (b) Excess thymidine: Excess thymidine blocks cells in S phase by feedback inhibition of several enzymes necessary for nucleotide conversion (17). A double block technique was used (18) in which a final concentration of 5 mM thymidine was added to a flask of exponentially growing L cells for 18 h, the block was released for 12 h (putting all cells in G_1) and then reapplied for an additional 18 h to arrest all cells at the start of S. (c) Serum starvation: Serum is essential for L-cell proliferation, with cells grown without serum block primarily at a point in G_1 ("G_0"; 19, 20). L cells were placed in a serum-free medium for 48 h, after which growth was stimulated by adding a final concentration of 10% FCS.

After synchronization, cells were counted and dispensed into microtiter plates or glass vials, IDS or control solution added, and DNA synthesis or intracellular cAMP levels measured at 2- to 4-h intervals thereafter.

cAMP Assay. Intracellular levels of cAMP were measured on sonicated cell samples using a commercial radioimmunoassay kit (Schwarz/Mann Div. Becton, Dickinson & Co., Orangeburg, N.Y.). 2×10^5 L cells were grown in 1 dram flat-bottomed glass vials in a final volume of 0.5 ml RPMI with 10% FCS along with the agents to be tested. At the end of the desired incubation period, the cells were centrifuged at room temperature (1,500 rpm for 2 min), the medium aspirated, and the cell pellet immediately frozen in ethanol-dry ice and stored at $-20°C$ until assay. To assay, the cells were resuspended in 1 ml of 0.05 N sodium acetate buffer, pH 6.2, boiled for 8 min to inactivate phosphodiesterase, sonicated for 2 min with a cup horn device (model W185, Heat Systems-Ultrasonics, Inc., Plainview, N. Y.), centrifuged (3,000 rpm for 15 min), and 300 μl of the supernate recovered for the radioimmunoassay. This method is considerably easier than conventional methods which include TCA precipitation and ether extraction of the material before assay. Table I shows, however, that our simplified method is quite reliable, as addition of exogenous cAMP, adenylate cyclase activators or phosphodiesterase inhibitors, or increasing the number of cells assayed, produce the appropriate increase in intracellular cAMP levels, and all activity is destroyed by phosphodiesterase.

Results

Effect of IDS on DNA Synthesis and Mitotic Index of Synchronized L Cells. As previously reported, IDS inhibits DNA synthesis in L cells. In an attempt to localize this activity to a particular point of the cell cycle, IDS was added to cells synchronized by various means and DNA synthesis quantitated by 2- or 4-h pulses therafter. L cells synchronized by mitotic shake-off and grown without IDS show a clearly defined peak of DNA synthesis, lasting from 10 to 18 h after mitosis, which represents S phase (Fig. 1 a). This S phase DNA synthesis is essentially completely absent in cells grown in the presence of IDS. Panel b shows that L cells synchronized by excess thymidine have

TABLE I
Validation of Cyclic AMP Assay on L Cells

Sample	pmoles of cAMP
5×10^4 cells	0.18, 0.17
" " " + 2 pmol cAMP	1.6, 1.7
" " " + 10 μg/ml PGE₁	1.5, 1.3
" " " + 5 × 10⁻³ M IBMX	0.38, 0.38
" " " + IBMX + PGE₁	2.9, 2.6
" " " (treated with phospho-diesterase)	<0.02
" " " + PGE₁ (treated with phosphodiester-ase)	<0.02
25×10^4 cells	0.60, 0.65

* L cells seeded in glass vials in medium containing 10% FCS 4 h before assay. Samples harvested 15 min before adding PGE₁ or IBMX. Addition of exogenous cAMP in line 2 was made immediately before harvest. For phosphodiesterase treatment, 10 μg/ml of 3,5 cyclic nucleotide phosphodiesterase (Sigma Chemical Co., St. Louis, Mo.) was added to sonicated cell suspensions for 1 h, after which the samples were boiled for 8 min to inactivate the enzyme, and assayed. Duplicate values are shown.
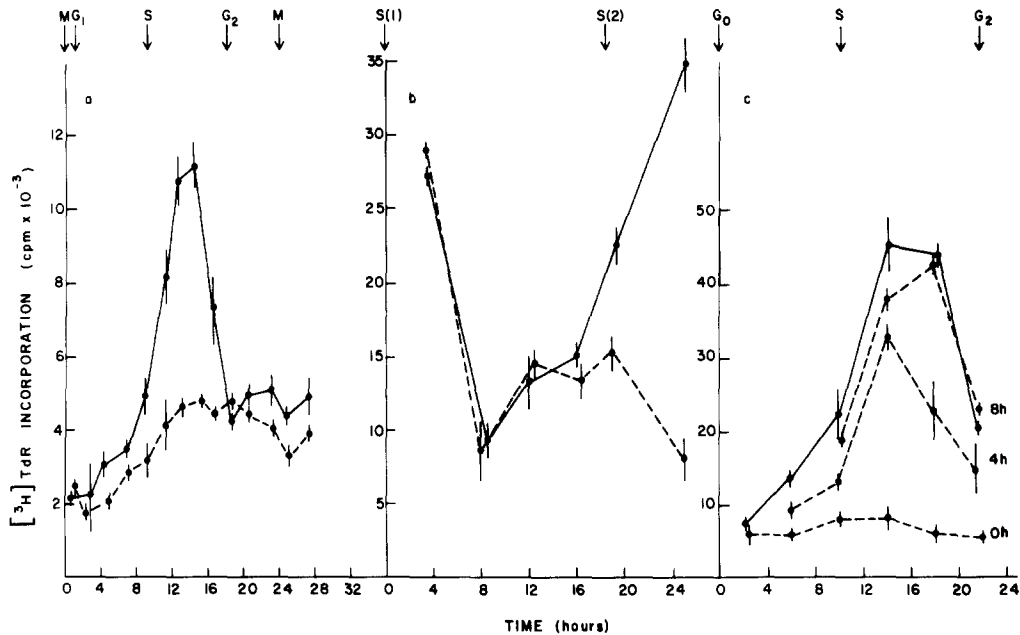
FIG. 1. Effect of IDS on DNA synthesis at various times in synchronized L cells. Control,
———; IDS, - - - -. (a) Mitotic shake-off; (b) excess thymidine; (c) serum starvation. Points
shown are at midpoint of sequential 2- (panel a) or 4-h (panels b and c) tritiated thymidine
pulses. Mean ± SE of duplicate microwells. Phases of the cell cycle corresponding to the
data are shown above.

initially high levels of DNA synthesis, which rapidly decline and then reach a
peak again about 20 h later. When IDS is present, there is no effect on the first
peak of DNA synthesis, but the second peak is significantly inhibited. Finally,
in panel c, L cells after serum starvation resume DNA synthesis after a 8- to
12-h lag period after the addition of serum. IDS added along with the serum
completely inhibited this DNA synthesis, but had little effect if added 4 or 8 h
after serum.

The timing of the IDS effect was further defined by experiments in which
IDS was added at various times after mitotic shake-off and S phase DNA
synthesis (Fig. 2) or mitotic index (Table II) were measured. In both instances
IDS was equally effective when added at 0 or 6 h after mitosis, but had little
effect if added at the start of S phase (10 h after mitosis). IDS added at 0 and
removed at 6 h was also without effect.

Effect of IDS on Intracellular cAMP Levels of Synchronized L Cells. Initial
evidence that IDS might act by altering intracellular cAMP levels was obtained
by studying the effect of isobutyl-methylxanthine (IBMX), a phosphodiesterase
inhibitor, and imidazole, a phosphodiesterase activator, on IDS-induced inhibi-
tion of DNA synthesis. As seen in Fig. 3, the slight inhibition of DNA
synthesis seen using a suboptimal dose of IDS is greatly potentiated by $5 \times$
10^{-4} M IBMX. Similarly, the inhibition produced by higher doses of IDS is
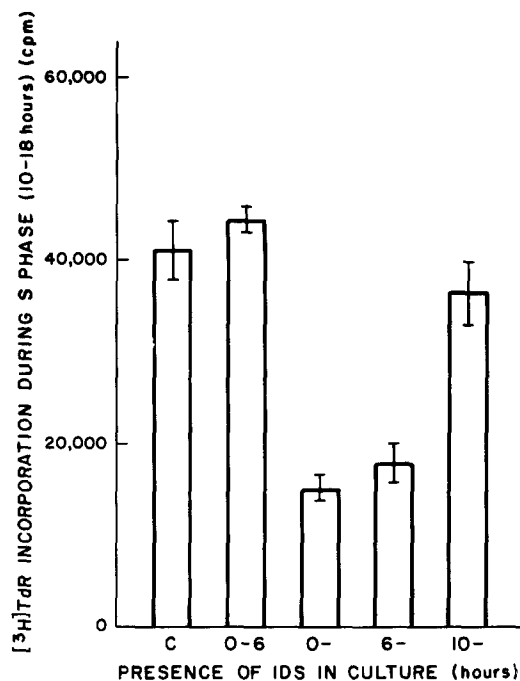partially overcome by 5×10^{-3} M imidazole. However, when we looked directly

FIG. 2. Effect of IDS added and removed at different times on DNA synthesis in L cells synchronized by mitotic shake-off. C, control supernate. Mean ± SE of duplicate micro-wells.

TABLE II
Effect of IDS on L-Cell Mitosis*

Presence of IDS	Mitotic index
h	
Control	42, 41
0–6	46, 41
0–	2, 1
6–	1, 4
10–	27, 30

* L cells synchronized by mitotic shake-off were grown in duplicate in microtiter plates containing IDS during the indicated times. Colcemid, 1 μg/ml, was added at 20 h, and the cells harvested and fixed at 24 h. A total of 300 cells were counted on aceto-orcein-stained slides. Mitotic index between 4 and 16 h was 7–9%.

at alterations in cAMP levels in unsynchronized L cells after treatment with IDS, no effect could be seen at any times between 2 min and 3 h after adding IDS, even in the presence of IBMX, which magnifies the action of any adenylate cyclase activator by inhibiting the breakdown of the cAMP once formed (data not shown). This is despite the fact that with our assay system other adenylate cyclase activators gave significant increases in cAMP levels (Table I).

One explanation for this lack of effect was that IDS only raised cAMP levels during the narrow portion of the cell cycle where it was effective in inhibiting
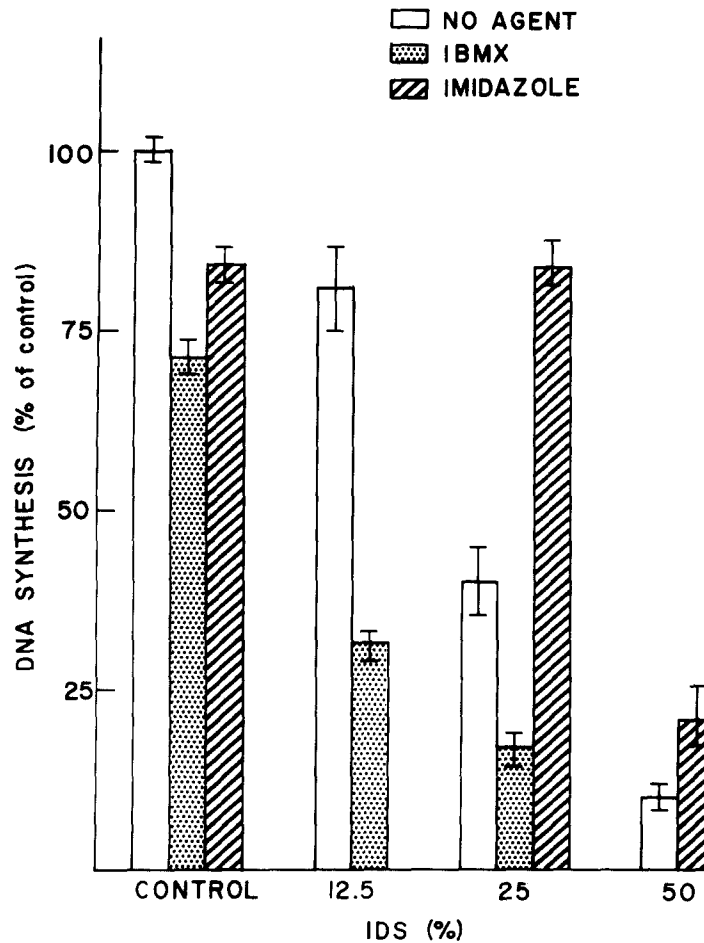
FIG. 3.   Effect of phosphodiesterase-active agents on L-cell DNA synthesis. L cells were cultured for 24 h with various concentrations of IDS and either IBMX (5×10^{-4} M) or imidazole (5×10^{-3} M) with a terminal 4-h pulse of tritiated thymidine. Mean ± SE of duplicate microwells.

DNA synthesis, and thus its effect would not be apparent in an unsynchronized population. Using L cells synchronized by the same three methods, we located a distinct time interval during which IDS will cause a significant, sustained elevation of cAMP. This time in all three methods of synchronization was approximately 4 h before S phase (Fig. 4), and thus corresponds with the phase of the cell cycle when IDS was effective in inhibiting DNA synthesis. This finding is further supported by the experiment in Fig. 5, in which L cells synchronized by mitotic shake-off were pulse-treated for 15 min with IDS in the presence of IBMX. IDS produced a threefold increase in cAMP at 8 h after mitosis, but had no effect at any other time tested.

Discussion

The results presented here show that IDS-induced elevation of intracellular cAMP level and inhibition of DNA synthesis occur only during the late G_1
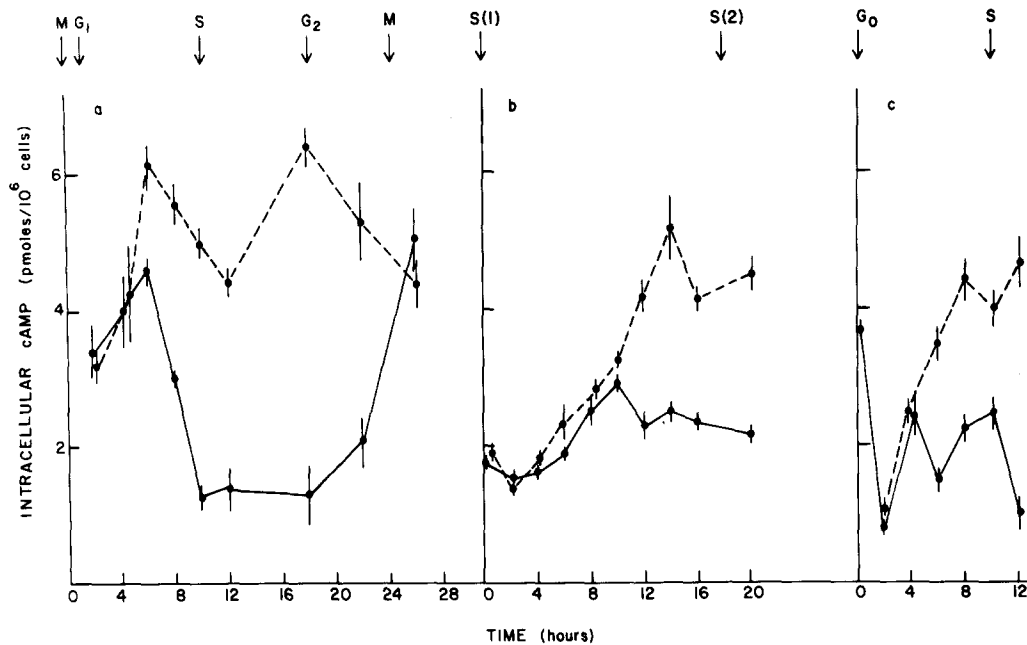
FIG. 4. Effect of IDS on cAMP levels at various times in synchronized L cells. Control,
——; IDS, - - - -. (a) Mitotic shake-off; (b) excess thymidine; (c) serum starvation. Mean ±
SE of triplicate cultures. Phases of the cell cycle correspond to those in Fig. 1.

phase of the cell cycle in L cells. Our previous studies (1) have shown that IDS action on mitogen-stimulated lymphocytes is also limited to late G_1 when a similar elevation of intracellular cAMP is seen (7). Thus, this observation is not a specific property of lymphocytes or mitogen-induced DNA synthesis but a general characteristic of IDS action. In both cell types the inhibition of DNA synthesis appears to be totally explicable by the increased cAMP levels. A correlation between high cAMP and low proliferation was previously shown in a variety of cell types, including fibroblasts and lymphocytes (21-23). In agreement with other investigators (24-26), we found striking variations of intracellular cAMP with the phase of the cell cycle, the highest levels occurring during early G_1 followed by a rapid fall and lower levels during late G_1 and S phase. Our data support the concept that the fall in cAMP during late G_1 may act as a "trigger" to initiate DNA synthesis because IDS acts essentially to prevent this fall. With regard to the mechanism behind this cell cycle specificity, we have preliminary evidence that receptors for IDS, discerned by the ability of cells to absorb IDS activity from a supernate, are present on lymphocytes only approximately 16 h after mitogen stimulation (unpublished observations).

The cell cycle specificity shown by IDS has interesting implications. cAMP itself plays a number of different roles in a wide variety of cell types, particularly as the second messenger for different hormones, in addition to its role in shutting off proliferation. An obvious question is how these diverse effects of cAMP can coexist within the same cell. This is particularly important in the case of IDS, which is produced by suppressor cells in situations where functional evidence of immunologic suppression is seen. It thus would be
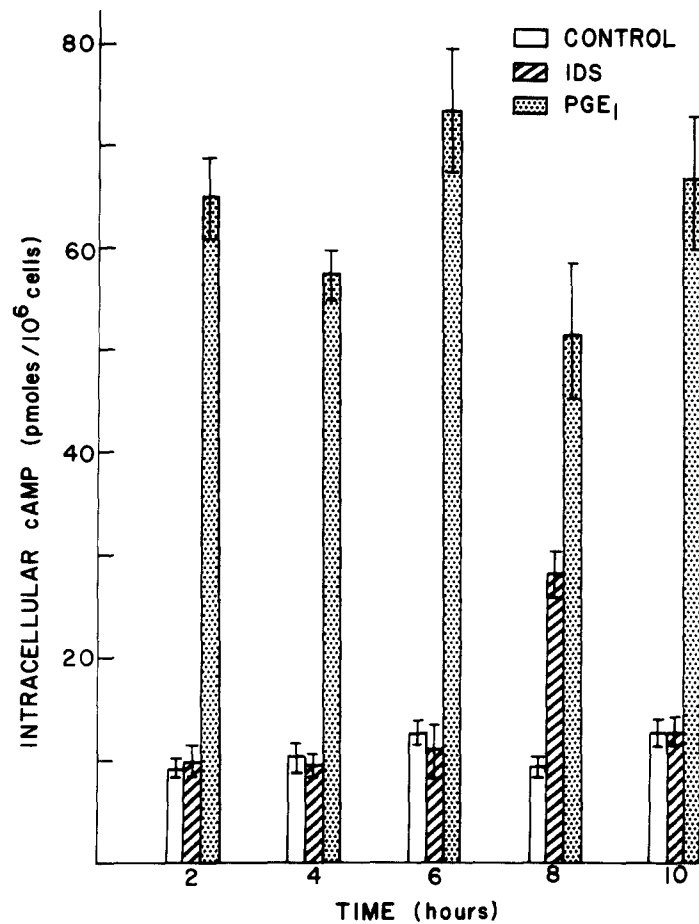
FIG. 5. Effect of IDS and PGE₁ (20 μg/ml) on cAMP levels in L cells synchronized by mitotic shake-off. Agents were added at the indicated time for 15 min along with 1×10^{-3} M IBMX to inhibit breakdown of newly formed cAMP, and tubes were then harvested for cAMP assay. Mean ± SE of triplicate cultures.

expected to bind to its target cell and raise cAMP levels only when the consequence would be suppression of that cell's function.

The phase of the cell cycle appears to play a key role in determining the different functional consequences of raising cAMP. The best understood example of this is the lymphocyte. There is considerable evidence that the resting (G_0) lymphocyte before being activated is in a stage of the cell cycle where cAMP exerts a positive growth stimulus, as opposed to the negative stimulus exerted by cAMP during the mitogenic response, as seen with IDS. Thus MacManus et al. (27, 28) have shown that increasing the intracellular cAMP concentration by a variety of agents causes DNA synthesis and mitosis in a subpopulation of thymocytes. Similarly in peripheral lymphocytes, Smith et al. (29) have shown that one of the first observable changes after adding mitogen is a transient increase in cAMP level. That this increase may be significant for the mitogenic response is suggested not only by its universal presence after stimulation with a wide variety of mitogens (21) but also by the fact that it

appears to be responsible for most of the other early events of the mitogen-activated lymphocyte (30, 31). Sulfhydryl compounds such as 2-mercaptoethanol, which increase mitogen-induced DNA synthesis, appear to do so by increasing the magnitude of this early rise in cAMP concentration (32).

Conversely, thymic hormones, which act on immature lymphocytes to make them more responsive to subsequent mitogen activation but inhibit ongoing mitogen responses, also appear to act by raising cAMP (33–35). As previously shown, dibutyryl cAMP and prostaglandin E_1 (PGE_1) can duplicate these effects of thymic hormone, but IDS cannot (36), suggesting another benefit to limiting IDS susceptibility to mitogen-activated mature lymphocytes.

Another persuasive example of cell cycle specificity is provided by Cloudman melanoma. Like most other cell lines, these cells show inhibition of DNA synthesis in the G_1 phase by added cAMP. They also show increased melanization (increased tyrosinase activity and melanin content) by either cAMP itself or melanocyte-stimulating hormone, a peptide hormone that raises cAMP in melanocytes and is thought to effect melanization thereby (37–38). However, melanocyte-stimulating hormone does not effectively inhibit DNA synthesis, and has been shown to bind to melanoma cells, raise cAMP levels, and increase tyrosinase activity only during the G_2 phase of the cell cycle. Thus melanoma cells have effectively dissociated the two arms of the action of cAMP–growth inhibition and increased melanization—by placing them in different phases of the cell cycle, so that melanocyte-stimulating hormone, which binds only during G_2 will cause melanization without inhibiting growth. Thus, by analogy, the cell cycle specificity of IDS action may be significant in limiting IDS action to suppression.

Summary

IDS inhibits DNA synthesis and mitosis of L cells only when present during the late G_1 phase of the cell cycle, as shown with L cells synchronized by a variety of methods. This corresponds well with earlier findings that IDS inhibits DNA synthesis in mitogen-stimulated lymphocytes when present between 16 and 24 h after adding mitogen. In both cell types, the inhibition produced by IDS appears to be totally the result of elevation of cAMP level. Thus, inhibitors of cAMP phosphodiesterase work synergistically with IDS, and activators of cAMP phosphodiesterase overcome the inhibition by IDS. This paper shows that IDS raises cAMP levels in L cells only within a narrow interval of the cell cycle, around 6–8 h after mitosis. This cell cycle specificity, which may be related to appearance of receptors for IDS only at discrete times, may be important in limiting IDS action to suppression, as elevated cAMP levels have a variety of other effects during other phases of the cell cycle.

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