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Isopentenyladenosine Stimulates and Inhibits Mitosis of Human Lymphocytes Treated with Phytohemagglutinin

Abstract. *The plant cytokinin isopentenyladenosine, a component of yeast and mammalian transfer ribonucleic acid, is both a potent inhibitor and stimulator of DNA synthesis, transformation, and mitosis of the phytohemagglutinin-stimulated lymphocyte. The stage of the cell cycle and the concentrations used are critical for these effects. The addition of isopentenyladenosine within the first 12 hours after phytohemagglutinin at a concentration above 10^{-6} molar results in inhibition, while lower concentrations (between 10^{-7} and 10^{-6} molar), added at 24 hours or later, have a stimulatory effect.*

Studies of biochemical events leading to and possibly controlling mammalian cell mitosis have been conveniently studied with the phytohemagglutinin-stimulated lymphocyte system. Mature human lymphocytes cultured in vitro generally do not synthesize DNA or divide; however, addition of phytohemagglutinin (PHA) results in a morphologic transformation to a lymphoblastoid cell capable of DNA synthesis and leads to eventual mitosis (*1*). These events are preceded by an increase in RNA and protein synthesis (*2*).

We have used this in vitro system in the study of the possible regulatory influence of transfer RNA (tRNA) and some of its minor bases. One of the minor bases, N^6-(Δ^2-isopentenyl)adenosine, is of unusual interest for the following reasons. (i) It is a potent plant hormone (cytokinin), stimulating both cell division and cell differentiation in plant systems (*3*). (ii) As a component of yeast (*4*) and mammalian (*5*) serine tRNA and yeast tyrosine tRNA (*6*) it may be essential for protein synthesis. Evidence has already been presented indicating that isopentenyladenosine is necessary for binding of seryl-tRNA to the ribosomal messenger RNA complex (*7*). (iii) It has antitumor activity in animals (*8*) and in man (*9*). We report here the results of our experiments demonstrating that, depending on conditions, isopentenyladenosine either inhibits human lymphocyte transformation and mitosis or has stimulatory effects.

Peripheral blood was obtained from healthy volunteers, and the plasma containing the leukocytes was separated from erythrocytes by sedimentation at 37°C. A portion (2 ml) of this plasma was suspended in 4 ml of Eagle's minimum essential medium containing 50 units each of penicillin, streptomycin, and glutamine (4 m*M*) gently mixed and incubated in 10-ml sterile screw-cap vials at 37°C without agitation. The leukocyte suspension had been adjusted to a final concentration of approximately 1×10^6 cells per milliliter. Phytohemagglutinin M (Difco), 0.3 mg, was added at the start of the incubation. Measurements of DNA synthesis were determined from the incorporation of deoxythymidine-2-^{14}C (0.5 μc/ml) on duplicate samples, and the results were averaged.

The cells were harvested by placing the vials in ice and immediately centrifuging at 4°C; the cell pellet was washed three times with 5 to 8 ml of cold isotonic saline. The cells were then lysed by repeated freezing and thawing, and the proteins and nucleic acids were precipitated by the addition of cold 5 percent perchloric acid. After 20 minutes, the samples were washed three times with 5 percent perchloric acid, dried, solubilized with NCS (Nuclear-Chicago), and counted in a Packard liquid scintillation counter (90 percent efficiency) after being transferred to counting vials containing 10 ml of toluene phosphor. Results for DNA synthesis were for 6×10^6 cells. Protein synthesis was determined in a similar manner with ^{14}C-leucine (0.5 μc/ml) added at the start of incubation. Total protein was measured by the method of Lowry *et al.* (*10*). The RNA synthesis was determined from incorporation of ^{3}H-uridine (1 μc/ml) added at the start of culture. The percentage of lymphocytes transformed with PHA was determined by microscopic examination of a Giemsa-stained, air-dried slide 48 hours after start of the incubation with PHA. The mitotic index was also measured at 48 hours by counting the number of cells in mitosis per 1000 nucleated cells (*11*). Chromosome preparations of the cells were made according to the technique of Moorhead *et al.* (*12*).

In initial experiments lymphocytes were separated from granulocytes by the nylon-column technique (*13*). The results of these experiments did not significantly differ from results obtained with unfractionated leukocytes. Therefore, we used unfractionated leukocytes. The addition of isopentenyladenosine (8 μmole/liter) at the initiation of the culture inhibited lymphocyte DNA synthesis, transformation, and mitosis (Fig. 1). The inhibition was not accompanied by visible chromosomal aberrations or cellular morphological alterations as judged by either electron or light microscopy. The addition of isopentenyladenosine at increasing intervals after PHA resulted in a decrease in the inhibition of transformation and mitotic figures (Fig. 1). Thus, the effects of isopentenyladenosine are critically dependent upon time of addition. Once PHA-stimulation has developed, and the cells are triggered to enter into S phase, inhibition by isopentenyladenosine is reduced. The addition of isopentenyladenosine (0.8 μmole/liter) at the start of incubation had no effect on the PHA-stimulated lymphocyte. However, later addition (24 to 36 hours after PHA) produced a twofold stimulation of DNA synthesis (Fig. 1C). In the absence of the triggering effect of PHA, isopentenyladenosine alone did not stimulate lymphocyte DNA synthesis.

In Fig. 2 the results of experiments showing the effects of varying concentrations of isopentenyladenosine on DNA synthesis, transformation, and mitosis are presented. In all cases, isopentenyladenosine was added 24 hours after PHA. Although there was variation in the concentrations of isopentenyladenosine required for stimulation and inhibition with lymphocytes from different donors, the curves in Fig. 2 are typical. The concentration of PHA was critical for obtaining stimulation with

isopentenyladenosine. Maximum stimulation with PHA was obtained by increasing the concentration (Difco PHA) from 0.3 to 1.0 mg per 6-ml culture or from 0.1 to 0.5 mg per 6-ml culture (Burroughs-Wellcome PHA). Under these circumstances, no further stimulation by isopentenyladenosine was found.

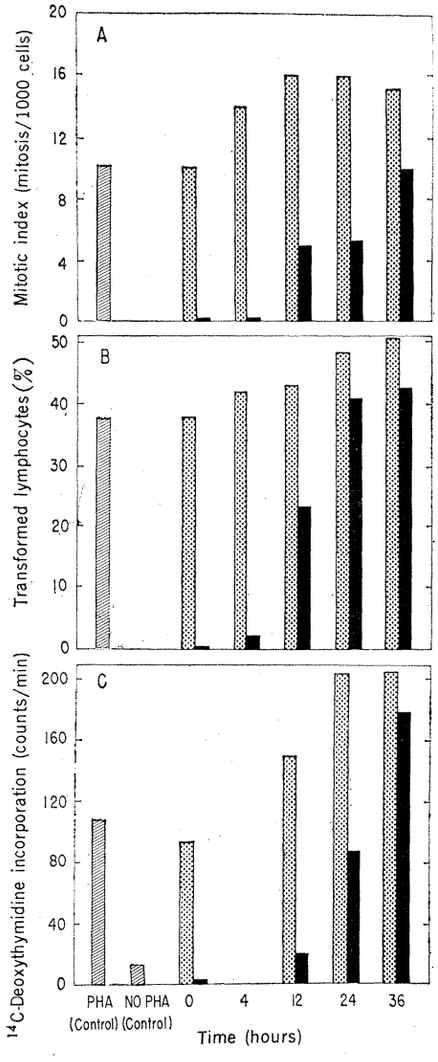

Fig. 1. Lymphocyte DNA synthesis (C), transformation (B), and mitosis (A) stimulated by phytohemagglutinin (PHA): time course of inhibitory and stimulatory effects of isopentenyladenosine. At the start of incubation PHA was added, and in each case the cells were harvested at 48 hours. Synthesis of DNA was determined by adding deoxythymidine-2-^{14}C (0.5 μc/ml) 24 hours after the start of incubation, the approximate time for the onset of maximum DNA synthesis (*19*), and measuring the radioactivity in the fraction precipitated in 5 percent perchloric acid. The isopentenyladenosine was added after PHA at the time indicated. Striped bars refer to control samples incubated with PHA in the absence of isopentenyladenosine or control samples incubated without PHA or isopentenyladenosine. Stippled bars refer to samples incubated with PHA and 0.8 μM isopentenyladenosine added at the indicated times. Solid bars refer to samples incubated with PHA and 8.0 μM isopentenyladenosine.

It was possible that the stimulatory effect of isopentenyladenosine was an artifact due to either a change in the intracellular content of deoxythymidine and thymine nucleotides or to a decrease in deoxythymidine degradation to thymine and deoxyribose-1-phosphate catalyzed by deoxythymidine phosphorylase (E.C.2.4.2.4). This enzyme is present in human lymphocytes but is particularly abundant in granulocytes (*14*). These possibilities were ruled out by the following observations: (i) similar results were obtained with a different precursor label (^{32}P); (ii) in addition to DNA synthesis, transformation (Fig. 1B) and mitosis (Fig. 1A) of the PHA-stimulated lymphocyte were also increased with 0.8 μmole/liter added at 24 or 36 hours; and (iii) no inhibition of the deoxythymidine phosphorylase activity of a cell-free system prepared from leukocyte extracts and assayed by methods previously described (*15*) could be detected with isopentenyladenosine.

Some evidence has been presented indicating that the effect of PHA may be at a membrane site (*16*). The possibility that the inhibitory effects of isopentenyladenosine were due to competitive effects with isopentenyladenosine at such a site, or that relatively high concentrations of isopentenyladenosine inactivated PHA, were considered and tested by the following experiments. (i) The effect of increasing concentrations of PHA was tested with one concentration of isopentenyladenosine. Increasing the amount of PHA up to sevenfold did not effect inhibition by isopentenyladenosine. (ii) Isopentenyladenosine was incubated with PHA either for 1 hour or for 4 hours, the sample was dialyzed against Eagle's minimum essential medium to remove isopentenyladenosine, and the PHA in the dialysis bag was tested for its stimulatory effects. No differences were found between PHA incubated with isopentenyladenosine and control PHA. (iii) Cells were incubated with isopentenyladenosine and PHA for a variable period of time. The cells were then collected, washed, and incubated again with PHA. The inhibition by isopentenyladenosine was not reversible. These results indicate that the inhibitory effects of isopentenyladenosine are not due to direct inactivation of PHA nor to competition at a PHA site on the cell membrane.

The inhibitory effects of isopentenyladenosine on DNA synthesis and subsequent mitosis were preceded by marked inhibition (70 to 100 percent) of RNA and protein synthesis. The mechanism for isopentenyladenosine inhibition of RNA synthesis might be the result of competition with adenosine, adenosine triphosphate, or deoxyadenosine triphosphate during RNA synthesis. However, the addition of adenine, adenosine, deoxyadenosine, or inosine even at concentrations sixfold greater than isopentenyladenosine did not diminish the inhibitory effects of isopentenyladenosine nor did pyrimidine nucleosides and deoxynucleosides (cytidine, deoxycytidine, and thymidine) decrease inhibition (*17*).

Several biologically interesting effects on isopentenyladenosine have now been reported, including its mitogenic and differentiating effects in plant systems and its antitumor effects. In some instances the results would appear paradoxical, as for example, the observa-

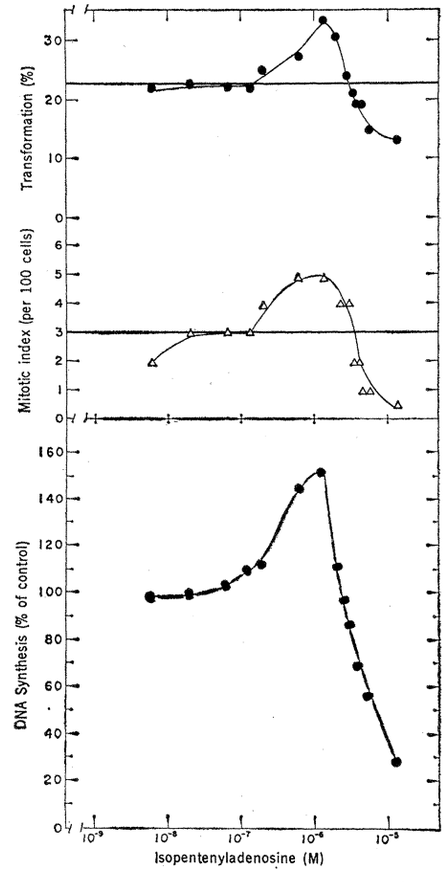

Fig. 2. The effects of graded amounts of isopentenyladenosine on lymphocyte DNA synthesis (lower panel), transformation, and mitosis (upper panel). The horizontal heavy lines refer to the control value (no isopentenyladenosine). The PHA (0.3 mg per culture) was added at the initiation of incubation, and isopentenyladenosine was added 24 hours later. The cells were harvested at 48 hours.

tions that isopentenyladenosine inhibits growth of undifferentiated precursors of white blood cells in tissue culture (myeloblasts) (*18*), while it appears to have stimulated cell division in vivo when used in the therapy of acute myeloblastic leukemia in man (*9*). These findings might be explained by the results reported in this communication demonstrating that isopentenyladenosine can have both growth-promoting and growth-inhibiting effects on human cells, depending on the concentration and on the particular stage of the cell cycle. The results of these experiments do not warrant extensive speculations on the mechanism or mechanisms of isopentenyladenosine inhibition and stimulation. However, some observations suggest that the primary effect of isopentenyladenosine is on RNA synthesis.

Frequently, in the treatment of human neoplasias, antitumor agents directed against the dividing cell are not effective because of a low rate of DNA synthesis accompanied by a relatively low incidence of mitosis. It is possible that isopentenyladenosine might be useful in some instances not only for its direct antitumor effects but to help trigger some cells to divide. In addition, inhibition of PHA-induced lymphocyte blastogenesis by isopentenyladenosine suggests that isopentenyladenosine has potential immunosuppressive properties.

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Renal Fructose-Metabolizing Enzymes: Significance in Hereditary Fructose Intolerance

Abstract. *In patients with hereditary fructose intolerance, which is characterized by deficient aldolase activity toward fructose-1-phosphate, fructose induces a renal tubular dysfunction that implicates only the proximal convoluted tubule. Because normal metabolism of fructose by way of fructose-1-phosphate requires fructokinase, aldolase "B," and triokinase, the exclusively cortical location of these enzymes indicates that the medulla is not involved in the metabolic abnormality presumably causal of the renal dysfunction.*

Invariably and apparently uniquely in patients with hereditary fructose intolerance, the sustained administration of fructose induces, within 30 minutes, a complex of proximal tubular dysfunctions that occur simultaneously and persist throughout the administration of fructose (*1, 2*). Renal medullary function appears unaffected (*2, 3*). These findings accord with the hypothesis that fructose induces a metabolic abnormality in the renal cortex but not in the renal medulla (*2*).

In hereditary fructose intolerance, the metabolic abnormality induced by fructose is initiated by cellular accumulation of fructose-1-phosphate (F1P) in those tissues deficient in aldolase activity toward this substance: liver (*4, 5*), kidney (*6*), and small bowel (*7*). These organs normally extract fructose briskly and convert it to glucose, predominantly by way of F1P and the triose products of its aldolase cleavage (*8, 9*). Fructokinase (E.C. 2.7.1.3) catalyzes the phosphorylation of fructose to F1P and is intact in patients with hereditary fructose intolerance (*4*). The "B" isoenzyme of aldolase ("liver aldolase") (E.C. 4.1.2.7) has strong cleaving activity toward F1P and, in keeping with the glucogenic capacity of liver and kidney, strong condensing activity toward dihydroxyacetone phosphate and D-glyceraldehyde-phosphate (*10, 11*). Triokinase (E.C. 2.7.1.28) catalyzes the phosphorylation of D-glyceraldehyde, one of the two aldolase cleavage products of F1P (*12*). Since fructokinase and aldolase B appear to occur only in the liver, kidney, and small bowel (*10, 13*), only these organs could accumulate F1P because of deficient aldolase activity. That the renal medulla might lack these fructose-metabolizing enzymes is suggested by the observation that rabbit renal medulla extracts fructose sparingly (*9*) and converts none to glucose and by the observation that aldolase activity toward F1P in the renal medulla is a small fraction of that in the cortex (*2*).

Table 1. Fructokinase, triokinase, and aldolase activities in mammalian tissues. The activities of fructokinase and triokinase are expressed as the number of micromoles of adenosine triphosphate formed per gram of protein per minute. The activity of aldolase is expressed as the number of micromoles of fructose-1,6-diphosphate (FDP) or fructose-1-phosphate (F1P) utilized per minute per milligram of protein, times 100. All the activities are expressed as the means ± the standard error of the means; the number of specimens assayed is given in parentheses.

Tissue	Enzyme activities				FDP/F1P*
	Fructokinase	Triokinase	Aldolase		
			FDP	F1P	
		Dog			
Kidney : cortex	4.9 ± 0.7 (5)	9.7 ± 2.0 (5)	14.5 ± 2.8 (9)	8.4 ± 2.0 (9)	1.8
Kidney : medulla	0 (5)	0 (5)	4.3 ± 0.6 (9)	0.2 ± 0.02 (9)	20.6
Muscle	0 (2)	0 (2)	101.9 ± 9.9 (5)	4.1 ± 0.5 (5)	25.3
Liver	2.7 ± 0.4 (2)	6.5 ± 0.9 (2)	8.5 ± 2.6 (5)	6.6 ± 2.1 (5)	1.4
		Human			
Kidney : cortex	4.7 (1)	10.0 (1)	3.2 (1)	2.5 (1)	1.3
Kidney : medulla	0 (1)	0 (1)	7.7 (1)	0.3 (1)	22.6

* Ratio of aldolase activity toward FDP and F1P.