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N^6-(Δ^2-*ISO*PENTENYL)ADENOSINE—EFFECTS UPON NUCLEIC ACID SYNTHESIS IN LYMPHOCYTES *IN VITRO* AND THE DEVELOPMENT OF IMMUNOLOGIC HYPERSENSITIVITY *IN VIVO*

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Abstract—The nucleoside, N^6-(Δ^2-*iso*pentenyl)adenosine (iPA), shown to be an integral constituent of certain transfer RNA's, has been found to inhibit the incorporation of uridine into RNA and thymidine into DNA. This assay *in vitro*, which uses phytohemagglutinin-transformed rat spleen cells, may reflect a measurement of immunosuppression. Although not as pronounced, inhibition has also been demonstrated in LM cells as well.

Evidence for an immunosuppressant property of iPA *in vivo* has been acquired, by using an immediate cutaneous hypersensitivity assay in rats.

Reference experiments were also conducted in parallel, testing actinomycin D and prednisolone, to ascertain their effects as immune-response depressants using both techniques described.

THE ABILITY of phytohemagglutinin to "transform" small human lymphocytes into rapidly proliferating lymphoblasts[1] is regarded with considerable interest from an immunological point of view. It has been demonstrated that this phenomenon is accompanied by accelerated protein and RNA synthesis followed by enhanced DNA formation.[2, 3] During the transformation process there is a concomitant induction of uridine kinase[4] as well as a transition in the type of RNA synthesized. The most prevalent species of RNA formed shortly (5–6 hr) after the addition of phytohemagglutinin has sedimentation properties identical with ribosomal and transfer RNA.[5] This system, thus, provides a facile one in which to study the effects of various immunosuppressants.

Although the role of "modified" nucleotides in transfer as well as ribosomal RNA is not altogether clear, it appears that the presence of these components in transfer-RNA, at least, is involved in the amino acid recognition process.[6] One such component in particular, N^6-(Δ^2-*iso*pentenyl)adenosine (iPA)* (Fig. 1, structure I) has been found in the tRNA from a variety of microorganisms, plants and mammalian tissues.[7–11] Sequence analysis has revealed the presence of iPA adjacent to the nucleotide triplets —IpGpA— in $tRNA^{Ser}$ (8) and —GpψpA— in $tRNA^{Tyr}$ (12) from yeast cells. Further analytical data by Peterkofsky tend to support the view regarding the occurrence of this compound in other acceptor tRNA species as well.[13] That iPA

* Abbreviations used are as follows: tRNA, transfer ribonucleic acid; $tRNA^{Ser}$, serine-acceptor transfer ribonucleic acid; $tRNA^{Tyr}$, tyrosine-acceptor transfer ribonucleic acid; iPA, N^6-(Δ^2-*iso*-pentenyl)adenosine (see Fig. 1, structure I); mRNA, messenger ribonucleic acid; PHA, phytohemagglutinin; UDR, uridine; TDR, thymidine; TCA, trichloroacetic acid.

is involved in regulating protein synthesis has been revealed by the recent studies of Fittler and Hall[11] in which selective chemical alteration of iPA of unfractionated yeast tRNA resulted in a diminished binding to the ribosome-mRNA complex without loss of acceptor activity. On the basis of recent studies it has been shown that mevalonate, a well known precursor of *iso*prenoid compounds,[14] will serve also as the donor of the N^6-*iso*pentenyl group in iPA.[13, 15, 16] Moreover, in this connection, iPA appears to be capable of affecting cholesterol biosynthesis *in vitro*.[17]

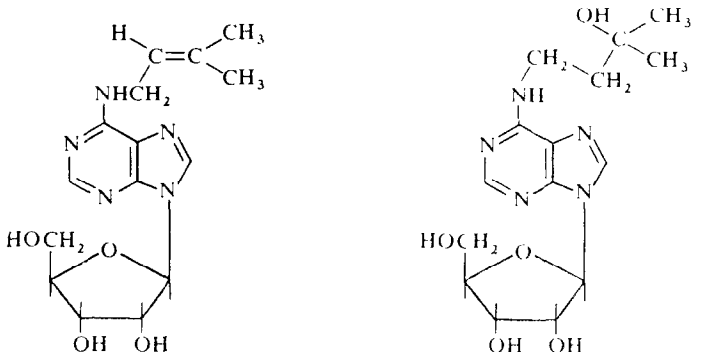

FIG. 1. Structure I, N^6-(Δ^2-*iso*pentenyl)adenosine; structure II, N^6-(3-methyl-3-hydroxybutylamino)-purine rubonuckoside.

In addition to its effects in cultured plant cells as a potent cytokinin,[7, 18, 19] iPA and a related derivative have been found as natural constituents of plant tRNA.[20]

This communication reports on the immunosuppressant property of iPA in "transformed" lymphocytes. Ancillary data are also presented on the effects of actinomycin D and prednisolone in these systems.

MATERIALS AND METHODS

A system similar to that described by Mishell and Dutton was used for cultivation of dissociated spleen cells *in vitro*.[21] In brief, spleens were removed from normal CFN rats (Carworth Farms) and teased apart in cold Hank's balanced salt solution using forceps. The suspended cells were transferred to sterile 12-ml tubes and placed in an ice bath for 10 min to allow cell debris to settle. The suspended cells were removed from the tubes using sterile 5-ml pipettes and transferred to graduated conical centrifuge tubes. After centrifugation (4°) at 1000 rpm for 10 min, the packed cell volume was diluted 1:160 with medium to produce a suspension containing approximately 1×10^7 cells/ml. The medium used in these experiments was an Eagle's minimal essential type, Spinner modification (Grand Island Biological Co.), supplemented with 200 mM glutamine per ml; minimal essential medium nonessential amino acids (100x), 1 ml; 100 mM sodium pyruvate, 1 ml; 5000 units each of penicillin and streptomycin; 10 per cent fetal calf serum (Rehatuin, Reheis Chemical Co.) per 100 ml medium.

One-ml aliquots of the spleen cell suspension ($1{\cdot}0 \times 10^7$ cells/ml) were placed in 16 × 100 mm sterile, disposable, glass culture tubes (Bellco Glass Inc.) and 0·01 ml of a 1:10 phytohemagglutinin-P (Difco) was then added to each. The designated amounts of iPA, actinomycin D (Merck & Co., Inc.) or prednisolone (Merck & Co., Inc.) were each dissolved separately in 1-ml portions of dimethyl sulfoxide (Matheson Coleman & Bell, spectro quality) and 0·001-ml amounts were added to each of five tubes containing the cell mixture. N^6-(Δ^2-*iso*pentenyl) adenosine samples used in these experiments were prepared synthetically according to Robbins *et al.*[10] or isolated from commercially available yeast in the manner described by Hall *et al.*[7] All tubes were incubated at 37° in a 5 per cent CO_2 atmosphere for 24 hr and then each was pulsed with either 5·0 μc (^{3}H)-thymidine (2·5 μg) (New England Nuclear Corp.) for 4 hr or with 5·0 μc (^{3}H)-uridine (2·5 μg) for 2 hr. At the end of this time, 1 mg of unlabeled carrier thymidine or uridine was added to each tube. Incorporation of the precursors was then determined upon the 5 per cent—TCA-precipitable material, using the procedure of Bollum.[22] The acid-insoluble material was taken up in 1·0 ml NCS reagent (Nuclear Chicago) plus 20 ml of standard scintillation solvent and counted in a Tri-Carb spectrometer (Packard).

In order to determine the activity of iPA on another cell line, LM cells, grown in suspension culture, were suspended at 2×10^5 cells/ml and tested in the same system as described for spleen cells omitting PHA.

A system using an immediate cutaneous hypersensitivity in the rat foot was used to study the activity of iPA *in vivo*. This experimentatal model is described and characterized elsewhere.[23] Male or female CFN rats weighing 160–180 g (Carworth Farms) were sensitized by injecting 0·1-ml suspension of 250 μg egg albumin (Calbiochem) in complete Freund's adjuvant (Difco Co.) into the right hind foot pad. Fourteen days later the rats were challenged in the left hind footpad with an egg albumin preparation containing 100 μg in 0·1 ml. Before and 1½ hr after challenge, the volume of the foot was determined by using a mercury displacement procedure as described by Van Arman *et al.*,[24] and the degree of swelling was determined. N^6-(Δ^2-*iso*pentenyl)adenosine at 25, 50 and 100 mg/kg and positive controls consisting of cyclophosphamide (12·5 and 25 mg/kg) (Cytoxan) or prednisolone (20 mg/kg) were suspended in normal saline and injected intraperitoneally 2 days before, on the day and 2 days after sensitization.

RESULTS

The uptake of (^{3}H)-uridine and (^{3}H)-thymidine into RNA and DNA, respectively (Table 1), was consistently inhibited in those cultured spleen cells which were stimulated with phytohemagglutinin (3 μg/ml) and treated with N^6-(Δ^2-*iso*pentenyl)adenosine (3 and 10 μg/ml). iPA was not, however, inhibitory in these cells at a concentration of 1 μg/ml (Table 1). Actinomycin D and prednisolone were used in parallel for comparative purposes. The former, a well-known cytotoxic agent, inhibits (^{3}H)-uridine and (^{3}H)-thymidine uptake in spleen cells incubated with antigen *in vitro* when tested at 0·002 to 20 μg/ml.[25] Recently, Lucas described repression of uridine kinase activity with actinomycin D using concentrations of 1 μg/ml.[4] We have confirmed these basic findings using 1 and 10 μg quantities of actinomycin D per ml of culture medium. Prednisolone, however, was only moderately active at the 10 μg/ml level with complete inhibition occurring at 30 μg/ml. This result would appear to conflict with recent

reports by Caron[26] and Tormey,[27] who have shown a 50–90 per cent inhibition of (^{3}H)-thymidine uptake by PHA-stimulated cells when a concentration of 10 μg/ml prednisolone was used. In both experiments, prednisolone was added 68–72 hr before the termination of the culture. Furthermore, Caron showed greatest inhibition when

TABLE 1. INHIBITION OF INCORPORATION OF [^{3}H]-URIDINE AND [^{3}H]-THYMIDINE BY PHYTOHEMAGGLUTININ-STIMULATED SPLEEN CELLS*

Agent	Concn (μg/ml)	Incorporation (cpm)		Inhibition of uptake (%)	
		(^{3}H)-uridine	(^{3}H)-thymidine	(^{3}H)-uridine	(^{3}H)-thymidine
iPA	10·0	2470	1038	85	92
	3·3	6450	3000	61	77
	1·1	15,200	9850	8	24
Actinomycin D	10·0	330	132	98	99
	1·0	823	261	95	98
Prednisolone	30·0	1649	1041	90	92
	10·0	10,723	9342	35	28
Control 1†	—	16,500	13,000	—	—
Control 2‡	—	6000	2000	—	—

* Twenty-four hr after the addition of phytohemagglutinin-P (5·0 μg/ml) to 1 × 10^7 spleen cells suspended in 1 ml of medium, the above compounds (in 0·001-ml vol.) were added and incubation at 37° continued for an additional 24 hr. Incorporation is expressed as radioactivity found in the 5 per cent TCA-precipitable material, after either a 2-hr pulse with 2·5 μc (5·0 μg) of [^{3}H]-UDR or a 4-hr pulse with 2·5 μc (5·0 μg) [^{3}H]-TDR. The per cent inhibition was calculated on the basis of PHA-stimulated controls. All cpms given reflect the average of 3 experiments and are correct to within ± 10 per cent.

† Phytohemagglutinin treated.

‡ No phytohemagglutinin treatment.

prednisolone was in contact with the cells for 48–72 hr. Since the cells are only maintained for 48 hr and the cytotoxic drugs are not added until the final 24 hr of incubation in our system, it is conceivable that a greater degree of inhibition may have been produced by using longer periods of culture.

Whitehouse has described a selective activity of indoxole (2,3-*bis*-*p*-methoxyphenyl-indole) against lymphocytes compared to H.Ep.-2 or U-cells.[28] In order to demonstrate whether iPA exhibited any specificity, 24-hr toxicity tests were performed on LM cells. In Table 2, it is seen that iPA (10 μg/ml) showed selective inhibition of

TABLE 2. INHIBITION OF (^{3}H)-URIDINE AND (^{3}H)-THYMIDINE INCORPORATION BY LM CELLS*

Agent	Concn (μg/ml)	Incorporation (cpm)		Inhibition of uptake (%)	
		(^{3}H)-uridine	(^{3}H)-thymidine	(^{3}H)-uridine	(^{3}H)-thymidine
iPA	10	10,800	23,000	64	8
Actinomycin D	1	1512	1257	95	95
Prednisolone	10	7508	6050	75	76
Control	—	30,000	25,000	—	—

* After incubating (37°) 2 × 10^5 LM cells in 1 ml of culture medium, together with the above compounds for 24 hr, the cells were pulsed with 2·5 μc (5·0 μg) of (^{3}H)-UDR for 2 hr or with 2·5 μc (5·0 μg) of (^{3}H)-TDR for 4 hr. Incorporation is expressed as radioactivity found in the 5 per cent TCA-precipitable material. All cpms given reflect the average of 3 experiments and are correct to within ± 10 per cent.

[^{3}H]-uridine uptake in contrast to the absence of any significant inhibition of [^{3}H]-thymidine incorporation. Actinomycin D, as expected, produced almost complete inhibition (1 μg/ml) in LM cells, while prednisolone at 10 μg/ml appeared more active against LM cells than lymphocytes. This is analogous to the results of Whitehouse using cortisol-21-aldehyde in H.Ep.-2 cells and lymphocytes.[28]

It is noteworthy that iPA is more inhibitory toward lymphocytes than LM cells with regard to precursor incorporation into both RNA and DNA (Tables 1 and 2). The most prominent feature, however, lies in the diminished inhibition exerted by iPA in the case of thymidine incorporation in LM cells. Studies are now in progress to assess the exact biochemical locus responsible for this variation.

Braun and Nakano have reported that iPA will produce a significant reduction in the number of spleen plaque-forming cells in mice stimulated by sheep red blood cells plus poly A and poly U.[29] This reduction was shown only in those reactions enhanced with the poly A and poly U, with little or no suppression of the normal immune responses.

The data presented in Table 3 show that iPA, when given for 5 consecutive days at the time of immunization, produced a small but significant reduction in the intensity of the hypersensitive reaction. Evidence presented elsewhere shows the classical

TABLE 3. IMMUNOSUPPRESSION *in vivo* BY N^6(Δ^2-*iso*PENTENYL)ADENOSINE ON IMMEDIATE HYPERSENSITIVITY IN THE RAT FOOT*

Treatment	Animals tested (No.)	Concn (mg/kg)	Increase in foot volume (cc)	Average inhibition of foot swelling (%)	Wt. change in treated animals (g)
iPA	6	25	0·63 ± 0·1†	21	+2·0
	12	50	0·58 ± 0·06	28‡	−2·5
	6	100	0·51 ± 0·01	36§	−17·0
Cytoxan	12	5	0·49 ± 0·06	39§	+1·0
	18	12·5	0·09 ± 0·04	89§	−5·0
	12	25	0·06 ± 0·02	92§	−11·5
Prednisolone	12	20	0·52 ± 0·05	35‡	−5·0
Saline (control)	12	—	0·80 ± 0·08	—	—

* All agents were suspended in normal saline and injected in the concentrations shown by the i.p. route for 5 consecutive days. The animals were sensitized with egg albumin on the third day of treatment and challenged in the foot pad 14 days later. Foot swelling was determined 2 hr after challenge by using a mercury displacement technique.

† Difference of the mean.

‡ $P = 0{\cdot}05$.

§ $P < 0{\cdot}01$.

anti-inflammatory drugs—Indocin, flufenamic acid and oxyphenbutazone—to be inactive in this model *in vivo* and demonstrates the specificity of the dosing schedule used for inhibition by known immunosuppressive agents.[23] This immunosuppressive activity of iPA (100 mg/kg) was equivalent to that of prednisolone (20 mg/kg), but much less than that of Cytoxan (12·5 or 25 mg/kg). Because of the severe weight loss during the course of treatment and a period of postinjection lethargy, iPA appeared to be more toxic than either prednisolone or Cytoxan.

Additional information concerning the dependency of suppressive activity upon the route of administration as well as the effects of iPA in other biological systems is currently being sought.

DISCUSSION

Various purine analogs, including 6-mercaptopurine, 6-thioguanine, 6-chloropurine and their corresponding ribonucleotides, exert multiple metabolic blocks which are responsible for many of the antineoplastic and immunosuppressive properties of these compound.[30-33] In contrast, N^6-(Δ^2-*iso*pentenyl)adenosine (iPA) (Fig. 1, structure I), shown herein to possess inhibitory activity in spleen lymphocytes, is unique in that it is a component found in a naturally occurring macromolecule.

The metabolism of iPA appears to involve formation of the intermediate, N^6-(3-methyl-3-hydroxybutylamino) purine ribonucleoside (Fig. 1, structure II), in a variety of mammalian and plant cell systems.[34, 35] In view of this finding it is unclear whether iPA *per se* is responsible for the observed inhibition of the tRNA methylases[36] found in *Escherichia coli* K_{12} or whether compound II or perhaps another intermediate is actually involved. Additionally, it is also unknown how iPA acts as an antitumor agent against such cell lines as Sarcoma-180 and certain human leukemic myeloblasts[37, 38] or what the mechanism of action is during the reduction of tumor cell counts in mice infected with Ehrlich carcinoma ascites cells.[39]

The new data presented here lucidly indicate that iPA is capable of preventing new RNA and DNA synthesis in spleen lymphocytes within 24 hr after the administration of phytohemagglutinin. The latter agent is known to induce elevated levels of nucleoside kinases, in particular uridine kinase.[4, 5, 40] In this connection, work is in progress to ascertain the more proximal biochemical locus of the action of iPA.

The results of inhibition reversal studies in *E. coli* suggest that iPA may inhibit cell growth by interfering with pyrimidine metabolism.[41] *De novo* purine synthesis is apparently not altered as indicated by the lack of reversal when preformed purines or their nucleosides are used.[38]

The occurrence of *iso*pentenyl adenosine proximal to the anticodon triplet in specific transfer RNA molecules and the possible relationship to its broad spectrum of biological activity still remain obscure. The antilymphocytic property of iPA *in vitro* and the mild suppressive activity *in vivo* may represent an important regulatory function of certain RNA constituents.

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