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Isoprinosine Augmentation of Phytohemagglutinin-Induced Lymphocyte Proliferation

J. W. HADDEN,* E. M. HADDEN, AND R. G. COFFEY

Laboratory of Immunopharmacology, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

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The need for agents designed to modify immune response in the treatment of patients with viral infection, immunodeficiency, or cancer prompted the present study on the mechanisms of action of isoprinosine, a compound developed for antiviral use and whose therapeutic activity may involve the immune system. The effect of isoprinosine on in vitro proliferation of human peripheral blood lymphocytes stimulated by phytohemagglutinin (PHA) and on lymphocyte levels of cyclic adenosine 3',5'-monophosphate and cyclic guanosine 3',5'-monophosphate was analyzed. Over a concentration range from 0.2 to 250 μg/ml, isoprinosine augmented PHA-induced proliferation; maximal stimulation was observed between 25 to 50 μg/ml. Isoprinosine in the absence of PHA had no effect on proliferation. The relative lack of effect of isoprinosine during a 90-min exposure and the lack of effect on lymphocyte cyclic nucleotide levels indicate that isoprinosine potentiates the PHA response by a mechanism different than a number of hormonal agents and such immunopotentiators as levamisole, polyadenylic-acid, and endotoxin. Further evaluation of isoprinosine as an immunopotentiator is indicated.

A clinical need exists for therapeutic agents designed to augment immune response in the treatment of patients with deficient or depressed resistance mechanisms. A number of immunopotentiators are under study for possible clinical use in patients with immunodeficiency, cancer, and viral infections (see reference 11 for review). Of the agents currently available for systemic viral infections, a number, including iododeoxyuridine (2) and cytosine arabinoside (20), have significant side effects resulting in suppression of host immune response; these side effects clearly limit the effectiveness of these agents (12). Since cellular immune response and interferon are thought to be central in the defense against most if not all virus infections, considerable current research attention has been focused on agents that augment cellular immune response (i.e., transfer factor, levamisole, polynucleotides, and bacterial adjuvants such as BCG) and induce or augment interferon-related mechanisms (e.g., exogenous interferon, polynucleotides, tilerones, and statalon).

One agent having reported antiviral effects (see reference 4 for review) is isoprinosine, the *p*-acetamidobenzoic acid salt of inosine dimethylamino-isopropanol (1:3 molar ratio). This compound has been shown in several clinical studies to produce a degree of enhancement of serum antibody response in patients with viral respiratory illness secondary to influenza or rhinovirus. With reference to cellular immune response, the effects of this agent to protect hamsters (5) and mice (4) from herpesvirus and influenza virus, respectively, are dependent upon mechanisms suppressed by antilymphocyte globulin or cortisone acetate, two well-known suppressants of cellular immune response. These experimental data suggest that the in vivo antiviral effects of isoprinosine may depend, in part, on an action to augment cellular as well as humoral immune response. The present study analyzes the effects of isoprinosine on an in vitro assay of the proliferative response of human peripheral blood lymphocytes stimulated by phytohemagglutinin (PHA) and demonstrates that isoprinosine significantly augments mitogen-induced lymphocyte proliferation. Analysis of the effects of isoprinosine on lymphocyte cyclic nucleotide levels indicates that, unlike a number of immunopotentiating influences, isoprinosine does not augment lymphocyte transformation through a direct action on these cyclic nucleotide regulatory pathways.

MATERIALS AND METHODS

Cells. Heparinized venous blood was obtained from 12 healthy adult donors. Suspensions of lymphocytes were obtained by Ficoll-sodium diatrizoate gradient centrifugation (21). Cells were washed in

Hanks balanced salt solution and spun at 150 × *g* to remove platelets. Final cell preparations were greater than 98% pure mononuclear cells (>85% lymphocytes by morphological criteria). Platelet contamination did not exceed 0.1% by weight.

Culture. Cells were cultured in Eagle minimal essential media including 10% human AB serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (Grand Island Biological Co., Grand Island, N.Y.) at a concentration of 10^5 lymphocytes/0.2 ml in microculture plates (Falcon Plastics, Oxnard, Calif.). Cultures were incubated in the presence and absence of PHA MR69 (Burroughs Wellcome, Research Triangle Park, N.C.) in a humidified atmosphere containing 5% CO_2 for 72 h. PHA when added was at final concentrations of 2.5, 5, or 10 mU/ml, representing submaximal, maximal, and supramaximal concentrations, respectively. Isoprinosine was kindly supplied by Newport Pharmaceuticals International (Newport Beach, Calif.) and was added to the cultures at various concentrations concomitant with PHA. In pulse exposure experiments, PHA and isoprinosine were incubated for 90 min with the lymphocytes. The lymphocytes were washed three times with Hanks balanced salt solution and incubated for 72 h. Proliferation was assayed by the incorporation of 0.5 μCi of [^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.) measured during the terminal 4 h of incubation. Cultures were terminated by a multiple automatic sample harvester (Otto Hiller Co., Madison, Wis.), and thymidine incorporation was assayed by liquid scintillation spectrometry. Cultures were performed in triplicate, and the data are presented as the averaged means of several different individual experiments plus or minus the standard error of the experimental means. Error terms, therefore, express interindividual variation rather than intraexperiment variation (<5% for triplicates).

Cyclic nucleotide measurements. Lymphocytes (10×10^6) were suspended in 1 ml of Hanks balanced salt solution in plastic Falcon tubes (12 by 117 mm). Lymphocytes were allowed to equilibrate for 1 h at 37 C. The experimental compounds were added in microliter volumes for various times, followed by termination with 0.5 ml of cold 30% trichloroacetic acid. Samples were frozen in dry ice and stored at −20 C. Samples were thawed, and 0.01 μCi of [^{3}H]cyclic guanosine 3′,5′-monophosphate (cGMP) (New England Nuclear) was added to determine recovery of each sample. Protein was removed by centrifugation and determined by the method of Lowry et al. (14). Trichloroacetic acid was removed from the supernatant by five extractions with 2 volumes of ether. Cyclic adenosine 3′,5′-monophosphate (cAMP) and cGMP were purified according to the protocol of Goldberg and Haddox (personal communication), involving sequential column separation with Dowex-1-formate (15), QAE-Sephadex A-25 formate (18), and Dowex-50 (H+). Recoveries were 40 to 60% for cGMP and 50 to 70% for cAMP, determined separately with ^{3}[H]cAMP containing 0.03-μCi samples. Aliquots treated with phosphodiesterase confirmed the purity (>95%) of all samples. cAMP was assayed by the method of Gilman (3), and cGMP was assayed by the radioimmunoassay method of Steiner et al. (19), using commercially available reagents (Collaborative Research, Inc., Waltham, Mass.). Acetylcholine (Sigma Chemical Co., St. Louis, Mo.) and prostaglandin E_1 (PGE_1; a gift from J. Pike, Upjohn Co., Kalamazoo, Mich.) were employed in these experiments to increase levels of cGMP and cAMP, respectively.

RESULTS

Effect of isoprinosine on PHA-induced lymphocyte proliferation. When isoprinosine was added to lymphocyte cultures in the absence of PHA, no effect on basal thymidine incorporation was observed. In the presence of an optimal mitogenic concentration of PHA (5 mU/ml), isoprinosine over a concentration range from 0.2 to 250 μ/ml augmented the PHA-induced proliferation (Fig. 1). At a concentration of 500 μ/ml isoprinosine had a variable effect: in the presence of an optimal concentration of PHA no stimulation occurred; however, in the presence of a supraoptimal concentration of PHA (10 mU/ml) inhibition resulted (data not shown). Over the concentration range of 0.2 to 250 μg/ml, isoprinosine stimulated PHA-induced proliferation in all individuals tested and peak stimulation averaged more than 50% greater than the PHA control. A double-peaked dose-response curve was observed, with peaks of stimulation at 1 and 50 μg/ml. Figure 2 represents a further characterization of the effect of low concentrations of isoprinosine to augment the response of lymphocytes at three concentrations of PHA (2.5, 5, and 10 mU/ml). Whereas low concentrations of isoprinosine augmented PHA response at all three concentrations, its effects were greatest at optimal PHA concentrations (5 mU/ml). The double-peaked dose-response suggested a complex drug action or a multiple drug action. Experiments (not shown) with the two components of the complex, inosine, and *N*,*N*, dimethylamino-2-propanol *p*-acetamidobenzoate incubated separately with PHA-stimulated lymphocytes indicate that the pattern of stimulation results from a potentiative interaction of the two components of the complex rather than additive, separate actions of the two components.

Effect of isoprinosine on early events of lymphocyte stimulation. (i) Pulse exposure. In an effort to discern whether isoprinosine exerts its effect on the early events of lymphocyte activation by PHA, a triggering event thought to occur within the first 3 h and probably the first hour of culture (9), pulse exposure experiments were conducted in which PHA and isoprinosine were incubated with lymphocytes for

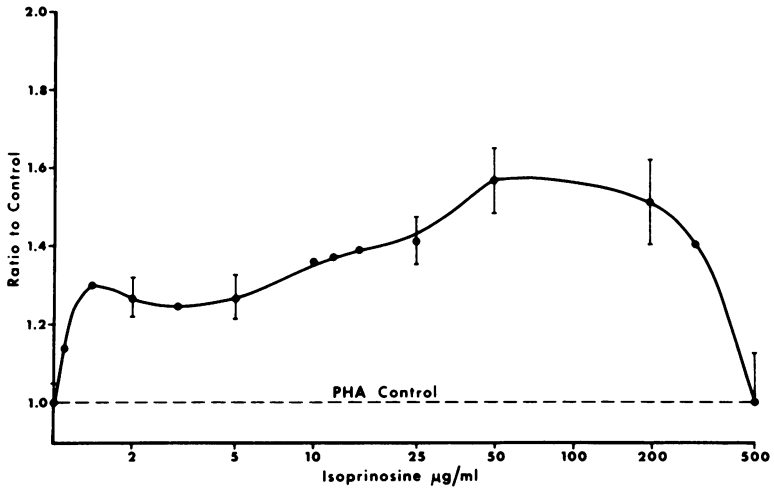

FIG. 1. *Effect of isoprinosine on PHA-induced lymphocyte proliferation. The effects of isoprinosine over a concentration range from 0.2 to 500 µg/ml were analyzed on lymphocyte proliferation induced by an optimal mitogenic concentration of PHA (5 mU/ml) in 12 individuals. The average incorporation of [³H]thymidine with PHA in these experiments was 41,000 counts/min per well. Because of variation among individuals the isoprinosine data are normalized as a ratio of the treated to the PHA response for that individual. Brackets represent the standard error of the means of the individuals studied and therefore represent the interindividual variation.*

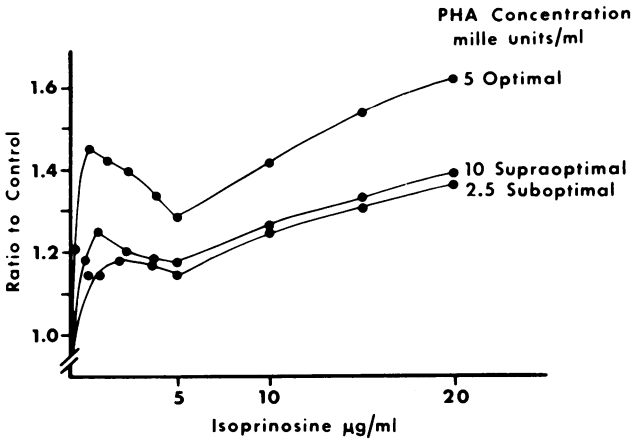

FIG. 2. *Effect of low concentrations of isoprinosine on PHA-induced lymphocyte proliferation. The results of one representative experiment of three employing different concentrations of PHA are depicted. Mean counts per minute of [³H]thymidine incorporation for PHA (2.5, 5, and 10 mU/ml) in this experiment were 38,000, 43,300, and 39,400, respectively. The effects of isoprinosine are represented as a ratio to the respective PHA control.*

90 min and washed prior to the 72-h culture, and were compared to results using continuous exposure (Fig. 3). After pulse exposure only a slight effect of isoprinosine to stimulate PHA transformation was observed compared to the continuous incubation. The parallel pattern of the dose-response curves suggests that the minor effects observed resulted from an incomplete action of isoprinosine, particularly at the higher concentrations. Pre-exposure of cultures to isoprinosine for 90 min prior to PHA addition (data not shown) resulted in a response somewhat less than co-incubation, indicating that, whereas isoprinosine action is not specifically on the initial triggering stage, its effects are probably limited in time during the culture period. This observation conforms to the estimated half-life of isoprinosine in culture of 1.3 h (T. Ginsberg et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, V73, p. 206). These experi-

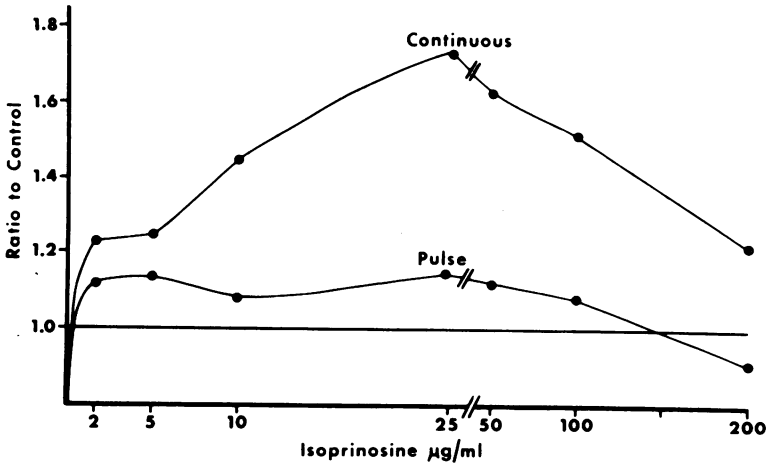

FIG. 3. *Effects of pulse exposure compared to continuous exposure to isoprinosine and PHA on lymphocyte proliferation. One representative experiment of three comparing a 90-min exposure to PHA (5 mU/ml) and various concentrations of isoprinosine to a continuous exposure with both. Mean counts per minute of [^{3}H]thymidine incorporation for PHA controls by pulse and continuous exposure were 22,600 and 26,400, respectively.*

ments indicate that isoprinosine action may result not from the recruitment of cells stimulated subliminally by PHA, but rather from the facilitation of proliferation of cells once triggered.

(ii) Cyclic nucleotide levels. Based upon recent observations that implicate the cyclic nucleotides as major biochemical pathways involved in the regulation of lymphocyte proliferative responses (cf. 9), the effects of isoprinosine on lymphocyte cyclic nucleotide levels were determined. A direct action on basal levels and an indirect one on the ability of other agonists to increase basal levels were measured to rule out effects either to stimulate the cyclic nucleotide cyclases or phosphodiesterases, respectively. Tables 1 and 2 show the results of two such experiments. No effects of isoprinosine at 2, 20, and 200 μg/ml were observed on basal levels of cGMP or cAMP or on acetylcholine or prostaglandin PGE_1-induced changes in these levels after a 1-h preincubation with 200 μg/ml. These experiments serve to exclude an effect of isoprinosine directly upon cyclic nucleotide generation or catabolism; however, they do not exclude an interaction with the cyclic nucleotides at the effector level.

TABLE 1. *Effect of isoprinosine on lymphocyte cGMP level*

Agent	Concn (μg/ml)	cGMP level as ratio to control[a]				
		2[b]	5	10	20	60
Isoprinosine	2	1.0	1.0	.9	1.2	
	20	1.2	1.1	1.0	1.1	
	200	1.1	1.0	1.2	1.0	1.1
Acetylcholine (ACH), 10^{-6} M			1.1	1.7		
Isoprinosine, 1 h, then ACH, 10^{-6} M	200		.9	1.5		
PGE_1, 10^{-5} M			1.0	1.0	.9	
Isoprinosine, 1 h, then PGE_1, 10^{-5} M	200		1.0	1.0	1.1	

[a] The control level of cGMP was 0.4 pmol per mg of protein.
[b] Time of incubation in minutes.

DISCUSSION

PHA-induced lymphocyte proliferation has provided a widely used technique for estimating immunocompetence of the cellular immune system and for studying the early events of lymphocyte activation. PHA induces proliferation of both T and B lymphocytes in human peripheral blood; however, the response of B lymphocytes to PHA in this system appears to be directly dependent upon, and in direct proportion to, T lymphocyte proliferation (7). Augmentation of the PHA response in this assay results from: (i) an increase in the relative proportion of T lymphocytes (i.e., an increase in the number of PHA-responsive cells); (ii) an increase in the number of cells responsive to a particular concentration of PHA (i.e., facilitated triggering); (iii) a magnification of the process once triggered (facilitated proliferation); or (iv) an increased recruitment involving macrophages and/or B lymphocytes. The pres-

TABLE 2. *Effect of isoprinosine on lymphocyte cAMP level*

Agent	Concn (μg/ml)	cAMP level as ratio to control				
		2[b]	5	10	20	60
Isoprinosine	2	1.0	.8	.9	1.0	
	20	.8	1.2	1.0	1.1	
	200	1.0	1.1	1.0	.9	1.0
PGE_1, 10^{-5} M		2.9		3.8	2.9	
Isoprinosine, 1 h, then PGE_1, 10^{-5} M	200	2.8		3.7	2.8	
Acetylcholine (ACH), 10^{-6} M			1.0	.9	.9	
Isoprinosine, 1 h, then ACH, 10^{-6} M	200		1.1	1.0	.9	

[a] The control level of cGMP was 41 pmol per mg of protein.

[b] Time of incubation in minutes.

ent experiments indicate that isoprinosine over a broad concentration range augments PHA-induced lymphocyte proliferation. The lack of effect of isoprinosine on the proliferation of lymphocytes not stimulated by PHA indicates that isoprinosine is not itself a mitogen nor an inducer of mitogenic factors (e.g., lymphocyte-produced blastogenic factor or macrophage-produced lymphocyte activating factor). The effect of isoprinosine on PHA stimulation in the absence of an effect on background indicates a potentiating interaction with PHA. The effect of isoprinosine on PHA stimulation was, correspondingly, maximal under conditions of PHA concentration which were maximal. The relative lack of effect of isoprinosine on events involved in the first 90 min of exposure to PHA (pulse exposure) and the lack of effects on lymphocyte cyclic nucleotide levels indicate that isoprinosine does not exert its effects on the early events of lymphocyte activation (10). On the basis of the foregoing it seems reasonable to interpret the action of isoprinosine within the perspective of facilitating the already initiated proliferative process [(iii) or (iv) above]. Such an interpretation is consistent with the only other observation concerning the possible biochemical action of isoprinosine, that is, to enhance polyribosome synthesis and function (5).

The rationale for examining isoprinosine within the perspective of cyclic nucleotide action in lymphocytes is provided by those observations that indicate that the initiation of lymphocyte proliferation involves cGMP, is augmented by humoral agents that increase lymphocyte levels of cGMP and inhibited by those that increase levels of cAMP. In contrast, proliferation once initiated is facilitated by agents that increase levels of cyclic AMP (see 8, 9 for review). A number of immunopotentiating agents shown to be active in vivo, including lipopolysaccharide (1, 17), levamisole (8a, 23), and polyadenylic acid·polyuridylic acid (6, 22) augment mitogen-induced lymphocyte proliferation in vitro and have been linked to cyclic nucleotides in their actions. These present experiments indicate the action of isoprinosine is different from these immunopotentiating agents and suggest that such action may be complementary to these agents, thus providing the basis for combined drug studies.

The present experiments demonstrate that isoprinosine has direct effects upon the in vitro proliferation of lymphocytes. Taken in conjunction with the evidence cited indicating effects in vivo upon immune response, they suggest that the further evaluation of the action of isoprinosine as an immunopotentiator, alone and in combination with other agents, is warranted.

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