Clin. exp. Immunol. (1983) **52**, 67–74.

Immunomodulation by isoprinosine: effects on *in vitro* immune functions of lymphocytes from humans with autoimmune diseases

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(Accepted for publication 5 November 1982)

SUMMARY

Isoprinosine (IPS) is a new anti-viral agent which appears to have immunomodulatory activities which include its ability to enhance the *in vitro* blastogenic responses of normal lymphocytes to mitogens. The present study compares the effects of IPS on the *in vitro* immune functions of peripheral blood mononuclear cells (PBMC) from systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) patients with its effects on PBMC from normal controls. Each mitogen (Con A, PHA or PWM) was used at its optimal concentration with a range of IPS concentrations (0–25 μg/ml). PHA-induced blastogenesis by PBMC from all three groups was enhanced by IPS at or above 5 μg/ml. The Con A-induced responses of SLE lymphocytes were significantly enhanced over controls by IPS ($P < 0{\cdot}02$ at 5 μg/ml) while those of RA lymphocytes were not. IPS had little effect on PWM-induced blastogenesis by RA lymphocytes but did enhance the blastogenic responses of SLE lymphocytes ($P < 0{\cdot}01$ at 5 μg/ml). In contrast, the characteristically high immunoglobulin synthesis by SLE lymphocytes was decreased by IPS. The mechanism responsible for these effects is not known but IL-2 production by patient lymphocytes *in vitro* which was low for both RA ($P < 0{\cdot}01$) and SLE ($P < 0{\cdot}02$) increased significantly ($P < 0{\cdot}05$) when SLE lymphocytes were cultured with IPS. These data identify IPS as an agent for the study of aberrant immune regulation in autoimmune diseases and suggest that it may have potential therapeutic value in SLE.

INTRODUCTION

Isoprinosine (IPS) is a new synthetic anti-viral agent composed of inosine and an organic salt at a 1:3 molar ratio (Ginsburg & Glasky, 1977). In early clinical trials with rhinovirus infected humans (Soto, Hall & Reed, 1973; Waldman & Ganguly, 1977), IPS increased the titres of circulating anti-viral antibody suggesting a humoral immunostimulatory effect. There is also evidence that IPS may enhance cell-mediated functions. For example, IPS amplified the *in vitro* blastogenic response of normal human lymphocytes to phytohaemagglutinin, concanavalin A, pokeweed mitogen and alloantigen (Hadden, Hadden & Coffey, 1976; Morin, Griscelli & Daguillard, 1979; Wybran, Govaerts & Appleboom, 1978).

Similar studies with murine splenocytes showed more dramatic potentiation of mitogenic responses by cells from aged animals than by cells from young animals (Ikehara *et al.*, 1981). In

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0009-9104/83/0400-0067$02.00

addition, a possible mechanism for these effects has been suggested by experiments in which IPS induced phenotypic maturation of murine nu/nu splenic T cells (Ikehara *et al.*, 1981; Renoux, Renoux & Degenne, 1979a). An even more specific immunoregulatory effect of the drug is implied by an increase in suppressor cell activity among murine lymphocytes treated with IPS (Renoux, Renoux & Gillaurin, 1979b).

Considering the apparent immunomodulatory effects of IPS, we wondered whether this drug might prove beneficial in the treatment of certain autoimmune diseases. The present study examines the *in vitro* effects of IPS on humoral and cell-mediated immune functions of lymphocytes from patients with systemic lupus erythematosus and rheumatoid arthritis.

MATERIALS AND METHODS

Subjects. All patients and control subjects participated in the study following informed consent in accordance with the regulations of the National Institutes of Health and the Committee on Human Experimentation of The University of Texas at San Antonio. The patient groups included 13 individuals with seropositive rheumatoid arthritis (RA, mean age = 53·5 years) and 16 with systemic lupus erythematosus (SLE, mean age = 40·8 years). Twenty-two healthy volunteers spanning both age ranges served as controls. Clinical findings in the patients with RA satisfied published criteria for definite (four patients) or classical (nine patients) disease (Ropes *et al.*, 1958). All RA subjects were taking salicylate or other non-steroidal anti-inflammatory drugs. In addition, four RA patients took prednisone on a daily basis (mean = 7·5 mg/day), two were receiving regular injections of gold thiomalate and four were taking D-penicillamine (250–500 mg/day). The diagnosis of SLE was made according to published criteria (Cohen *et al.*, 1971). Therapy in these patients included salicylates or non-steroidal anti-inflammatory drugs alone in 10, corticosteroids in seven (mean dose 7·3 mg/day, range 2·5–15 mg/day), plaquenil four and immuran in one.

Separation of the cells. Peripheral blood mononuclear cells (PBMC) from patients and normal controls were obtained from heparinized venous blood by discontinuous Ficoll-Hypaque gradient centrifugation (Böyum, 1968). PBMC were resuspended in RPMI 1640 (GIBCO, Grand Island, New York, USA) culture medium containing 5% heat-inactivated fetal calf serum (FCS, GIBCO) glutamine (2 mM), penicillin (100 units/ml) and streptomycin (0·1 mg/ml).

Preparation of drug solution. Isoprinosine (inosine-*N*,*N*-dimethyl-amino-2-propanol para-acetamidobenzoate, Lot No. AA1056), a gift from Newport Pharmaceuticals International Inc. (Newport Beach, California, USA) was dissolved fresh daily in RPMI 1640 culture medium at the concentration of 100 mg/ml. This solution was filter sterilized (0·45 μm Millipore Corp., Bedford, Massachusetts, USA) and diluted serially prior to use.

Mitogen-induced blastogenesis. PBMC (1×10^5) were suspended in RPMI culture medium and incubated in round bottom microtitre wells (Flow Lab., McLean, Virginia, USA) with optimal concentrations of each mitogen (phytohaemagglutinin, PHA-P, 1 μg/ml, Burroughs-Wellcome, Greenville, North Carolina, USA; concanavalin A, Con A, 5 μg/ml, Sigma Chemical Co., St Louis, Missouri, USA and pokeweed mitogen, PWM, 1:100 dilution, GIBCO) with or without the drug solution. Incubation was continued for 4 days at 37°C in a humidified atmosphere of 5% CO_2 with addition of 1 μCi of ^{3}H-thymidine/well (^{3}H-TdR, Schwarz-Mann, Orangeburg, New York) 18 h before harvesting. The effect of IPS on mitogenic responses was calculated according to the following formula:

$$\text{Response ratio} = \frac{\text{ct/min (PBMC + IPS + mitogen)}}{\text{ct/min (PBMC + media + mitogen)}} = \frac{\text{ct/min with drug}}{\text{ct/min without drug}}.$$

IL-2 production. PBMC (2×10^6/2 ml) were suspended in RPMI culture medium with PHA-P at 1 μg/ml and with IPS concentrations varying from 0 to 10 μg/ml (Gillis *et al.*, 1981). They were cultured at 37°C for 24 h in flat bottom tissue culture wells (No. 3506 Costar, Cambridge, Massachusetts). Culture supernatants were harvested by centrifugation (200*g*) for 10 min followed by filter sterilization (0·45 μm). The culture fluids were assayed for IL-2 activity as described by

Dauphinee *et al.* (1982) by their addition (at final dilutions of 1:2, 1:4, 1:8) to murine splenocytes (1×10^5 cell/ml) which had been activated for 48 h with Con A and washed with α-methyl mannoside. ^{3}H-thymidine was added during the last 18 h of the 48 h assay culture. The activity of IL-2 in a culture supernatant was expressed in units by comparison with the ^{3}H-thymidine uptake induced by a standard IL-2 preparation. The specificity of this system for IL-2 was shown in control experiments which exhibited no effect on blast cell proliferation from PHA-P carried over in the culture supernatants.

IL-2 response. PBMC from patients or normal controls were incubated in the presence of Con A (10 μg/ml) for 48 h and washed with RPMI 1640 containing 10 μg/ml of α-methyl mannoside. They were then incubated in culture media containing α-methyl mannoside, a standard IL-2 preparation and a range of IPS concentrations. ^{3}H-thymidine was added for the final 18 h of the 3 day cultures as described above.

Ig synthesis. PBMC (1×10^5/well) were suspended in RPMI culture medium and incubated with IPS concentrations ranging from 0–25 μg/ml. These were then cultured in the presence or absence of PWM at its optimal (1:100 dilution) concentration. After 7 days at 37°C in a humidified atmosphere of 5% CO_2, the supernatants were harvested by centrifugation. Aliquots of each supernatant were separately analysed for IgG and IgM concentrations by a solid phase radioimmunoassay using class specific rabbit $F(ab')_2$ anti-human gammaglobulin (Mariotta *et al.*, 1982).

Statistical analysis. Statistical comparisons between measurement means were accomplished by the two tailed paired sample *t*-test.

RESULTS

Effects of IPS on mitogenic responses

As shown in Fig. 1, isoprinosine had a biphasic effect on PHA-induced blastogenesis of normal PBMC. At the lowest drug concentration tested (0·5 μg/ml), the uptake of ^{3}H-thymidine was decreased slightly when compared with no added drug (response ratio = 1·0) while higher concentrations (≥ 5 μg/ml) substantially enhanced uptake by normal cells. Thus the response was

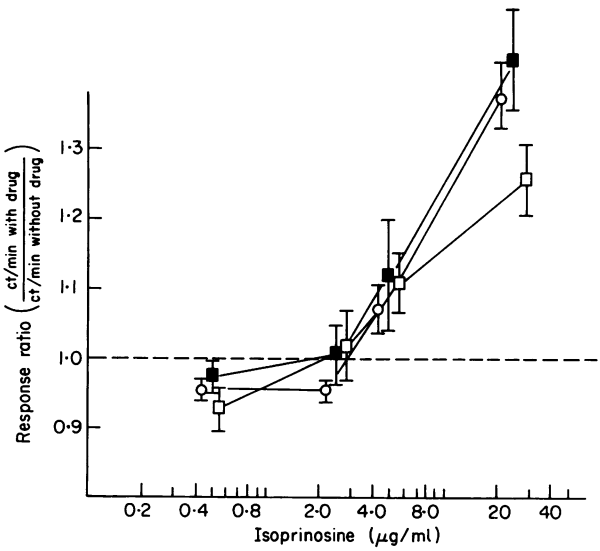

Fig. 1. Effect of isoprinosine on PHA-induced blastogenesis. PBMC from SLE (■, $n = 16$), RA (□, $n = 13$) and normal (○, $n = 22$) subjects were cultured with the optimal mitogen concentration and four concentrations of drug. The ordinate compares the responses with and without drug. Lymphocyte blastogenesis of all three subject groups was augmented by 25 μg/ml isoprinosine.

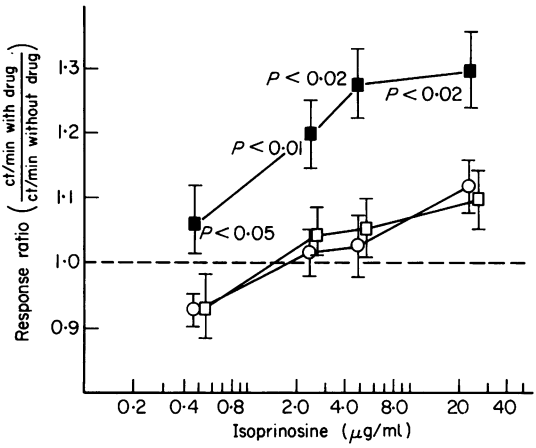

Fig. 2. Effect of isoprinosine on Con A-induced blastogenesis. PBMC from patients and controls were cultured with the optimal mitogen concentration and four concentrations of isoprinosine. The co-ordinates are similar to Fig. 1. Slight stimulation of blastogenesis occurred with RA and controls at 25 μg/ml isoprinosine. The response of SLE lymphocytes was enhanced significantly at all concentrations of isoprinosine tested. The symbols and numbers involved are the same as Fig. 1.

significantly greater ($P<0{\cdot}01$) at 25 μg/ml IPS than at or below 2·5 μg/ml. The responses of leucocytes from the two patient groups were similar and did not significantly differ from those of the normal controls. A less dramatic effect on normal PBMC blastogenesis was observed with Con A (Fig. 2). Con A-induced blastogenesis of lymphocytes from SLE patients in the absence of IPS (mean ±s.e. (mean) 18,148±2,031 ct/min) was lower than with normals (22,603±2,997 ct/min) although the difference was not statistically significant. When IPS was present in the cultures, the response of SLE lymphocytes to Con A was substantially enhanced even at the lower concentrations (2·5 μg/ml, $P<0{\cdot}01$; 5 μg/ml, $P<0{\cdot}02$ and 25 μg/ml, $P<0{\cdot}02$). The result of this effect was normalization of the response of SLE lymphocytes to Con A.

IPS also significantly increased the response of SLE PBMC to PWM mitogen (Fig. 3). By contrast, RA lymphocyte responses were slightly inhibited when compared with controls.

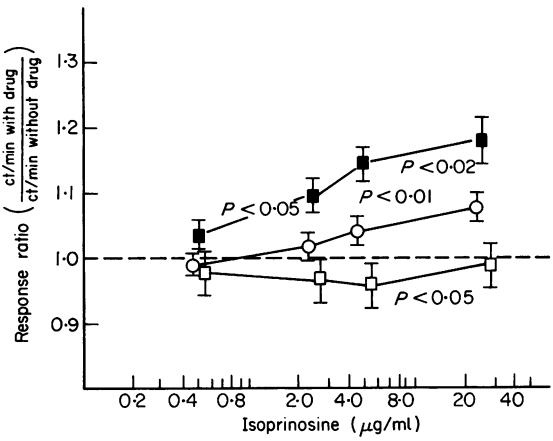

Fig. 3. Effect of isoprine on PWM-induced blastogenesis. PBMC from patients and controls were cultured with the optimal mitogen concentration and four levels of isoprinosine. The coordinates are similar to Fig. 1. Slight augmentation of the SLE lymphocyte response is seen at the higher drug concentrations. The symbols and numbers are the same as Figs 1 & 2.

Effects of IPS on production of and response to IL-2

PBMC from SLE, RA and normal subjects were stimulated by PHA-P to produce IL-2 in the presence or absence of IPS. Without IPS, SLE and RA lymphocytes produced significantly less IL-2 than did normal lymphocytes (Table 1). At 5 μg/ml, IPS stimulated a significant increase in IL-2 production by normal PBMC while a similar trend with RA and SLE lymphocytes nearly normalized their response but did not meet significance criteria.

Responsiveness of patient and control lymphocytes to IL-2 was measured by preincubating them with Con A for 2 days followed by exposure of their Con A-induced blasts to various dilutions of a standard IL-2 preparation. In the absence of IPS, IL-2 responsiveness of RA lymphocytes was significantly lower than that of the controls ($P < 0{\cdot}02$). The mean response with SLE leucocytes was also lower but not significantly different from normal. IPS had no significant effect on the response of normal or SLE PBMC to IL-2 (Table 2). It also failed to normalize the decreased responses of RA lymphocytes.

Table 1. Effect of isoprinosine on IL-2 production*

	IL-2 units (mean ±s.e. (mean))		
Isoprinosine (μg/ml)	0	2·5	5
SLE ($n=8$)	6·0±1·08	6·1±1·21	8·2±1·50
RA ($n=9$)	5·7±1·00	6·0±0·60	7·7±1·90
Normal ($n=15$)	8·0±0·18	9·0±0·03	9·5±0·05

* Assay of IL-2 containing culture fluids at final dilution of 1:40.

Statistical comparisons of RA ($P<0{\cdot}01$) and SLE ($P<0{\cdot}02$) at IPS = 0 with normal at IPS = 0.

Statistical comparison with normal at IPS = 0; $P<0{\cdot}001$.

Table 2. Effect of isoprinosine on IL-2 response

	^{3}H-thymidine incorporation (ct/min $\times 10^{-4}$)*			
Isoprinosine (μg/ml)	0	2·5	5	25
SLE ($n=16$)	2·05±0·18	1·73±0·12	1·88±0·07	1·80±0·10
RA ($n=13$)	1·55±0·25†	1·32±0·06	1·52±0·12	1·44±0·07
Normal ($n=22$)	2·50±0·24	2·23±0·13	2·38±0·19	2·33±0·16

* All values expressed as mean ±s.e. (mean).

† $P<0{\cdot}02$ compared with normal at IPS = 0.

Effects of IPS on in vitro *Ig synthesis*

PBMC from each subject were cultured for 7 days in the presence or absence of PWM and varying concentrations of IPS. The culture supernatants were harvested and tested for IgG and IgM by radioimmunoassay. Without PWM, SLE lymphocytes produced significantly more IgG and IgM than did normal lymphocytes (Table 3). IPS tended to reduce the enhanced Ig synthesis by SLE lymphocytes while it had no effect on Ig synthesis by RA and normal lymphocytes. When PBMC were stimulated with PWM, lymphocytes from both RA and SLE produced significantly less IgG and IgM than did normals. IPS tended to further decrease Ig synthesis by SLE lymphocytes incubated with PWM.

Table 3. Effect of isoprinosine on *in vitro* immunoglobulin production

	IgG			IgM		
Isoprinosine (μg/ml)	0	2·5	25	0	2·5	25
*Spontaneous Ig production**						
SLE ($n=9$)	100±24†	79±9	83±6	18±4§	14±2	16±2
RA ($n=10$)	63±16	62±4	61±4	10±3	10±1	12±1
Normal ($n=15$)	39±6	30±4	39±4	8±2	9±1	10±1
*Pokeweed mitogen-induced Ig production**						
SLE ($n=9$)	343±89§	298±27	305±14	60±26‡	55±7	52±4
RA ($n=10$)	312±92‡	340±22	328±13	52±22†	62±4	57±6
Normal ($n=15$)	676±94	804±68	730±54	138±17	160±14	148±11

* All values expressed as mean ±s.e. (mean).

Statistical comparisons of RA and SLE with normal at IPS=0; †$P<0{\cdot}01$; ‡$P<0{\cdot}02$; §$P<0{\cdot}05$.

DISCUSSION

Previous investigations have documented an IPS-induced enhancement of mitogenic responses by human (Hadden *et al.*, 1976; Morin *et al.*, 1979) and murine (Ikehara *et al.*, 1981) lymphocytes. Our current study confirms those findings using normal PBMC but also extends them to patients with autoimmune disease.

The IPS effect on PHA-induced blastogenesis of normal cells was of the same magnitude as observed by Hadden *et al.* (1976). With this mitogen, the responses of the patients' lymphocytes did not deviate significantly from normal. In contrast, the enhancement by IPS of Con A-induced SLE lymphocyte blastogenesis was substantially greater than observed with normals. Since the Con A-induced blastogenesis of SLE lymphocytes was lower than normal in the absence of drug, this IPS-induced effect normalized the lupus lymphocyte response. The same trend was seen with stimulation of blastogenesis by PWM.

PHA and Con A are known to exhibit some selectivity in augmenting proliferation of helper and suppressor T cells respectively, (Haynes & Fauci, 1977; Dosch, Schuurman & Gelfand, 1980). While both functional populations respond to both mitogens, the relative proliferative response of suppressor T cells to Con A is greater than with PHA (Reinherz *et al.*, 1981). Thus, the data presented in Fig. 2 suggest a selective enhancement of suppressor cell proliferation by IPS in SLE, a disorder known to exhibit deficient suppressor cell activity (Sagawa & Abdou, 1978). Such an enhancement of suppressor cell generation by IPS has been previously documented in a normal murine model after 3 days of pre-culture with the drug (Renoux *et al.*, 1979c). That suppressor activity was associated with T cells and was capable of suppressing an allogeneic mixed leucocyte reaction by over 30%.

If such a suppressor cell population is being generated by *in vitro* incubation of human cells with IPS, it is not apparent in the mitogenic responses which increase progressively with drug concentration. If suppressor cell generation requires 3 days of pre-culture however, the mitogenic responses which are assayed on days 3–4 may no longer be responsive to suppression. By contrast, the decrease in Ig synthesis by lupus lymphocytes seen with IPS over a 7 day culture could be due to active suppression. That decrease represents a drug-induced trend toward normalization of the excessive immunoglobulin synthesis by lupus lymphocytes (Russell *et al.*, 1980).

The mechanism for the immunomodulatory effects of IPS remains uncertain (Wybran *et al.*, 1978; Renoux *et al.*, 1979a, 1979b; Vecch, Sironi & Spreafico, 1978; Hadden, 1978). At the cellular level, there is evidence for an increase in nucleocytoplasmic transport of messenger RNA (Ronsen &

Gordon, 1976). Dosch & Shore (1982) have recently proposed that defects in lymphokine production are responsible for aberrant proliferation of regulatory T cells, particularly the suppressor population. The blastogenic response of normal lymphocytes to PHA is mediated by sequential production of IL-1 and IL-2 (Larsson, 1981; Bonhard, Yasaka & Jacobson, 1979; Lopez-Botet *et al.*, 1982). An IPS stimulated increase in IL-2 production was found in our study and could in part account for the drug's immunopotentiating effects.

The trend towards normalization of the immunological abnormalities in SLE suggests theoretical benefit for IPS in the treatment of SLE. Already, human clinical trials of this drug in autoimmune disease have involved patients with RA and subacute sclerosing panencephalitis (Wybran, Famaey & Appleboom, 1981; Haddad, 1980). Those studies are sufficiently promising to indicate cautious optimism for its usefulness in RA. In combination with interferon, isoprinosine has also induced a substantially prolonged survival for NZB/NZW F_1 mice with murine lupus (Sergiescu *et al.*, 1981) and of Swiss mice inoculated with sarcoma tumour cells (Cerutti, Chan & Schlumberger, 1979). Clearly, further study of this drug is indicated to fully characterize its immunopharmacological potential.

Supported in part by a grant from Newport Pharmaceuticals International, Inc., Newport Beach, California.

The authors wish to recognize the expert technical assistance of Gil Vipraio and the clerical skills of Beverly Keeton who typed the manuscript.

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