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A Double-Blind Clinical Trial of the Effects of Inosine Pranobex in Immunodepressed Patients with Prolonged Generalized Lymphadenopathy

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In a double-blind clinical trial, 61 immunodepressed males with persistent generalized lymphadenopathy (PGL) received one of two doses (1 or 3 g/day) of the immunomodulating drug inosine pranobex (INPX) or placebo for a period of 28 days. In the high-dose group, clinical improvement was reported by 11 of 21 patients (52%), within 5 months of the cessation of treatment. In contrast, 3 of 19 patients (16%) in the placebo group reported clinical improvement by that time. Patients receiving 3 g/day INPX showed a significant increase in NK cell activity by Day 14 and this elevation was still evident at the last follow-up examination 1 year after treatment. Increases in total T lymphocytes (T-11) and the percentage of T helper cells (T-4) were also observed. These responses were delayed and reached their peaks 2 months after the termination of drug treatment. The kinetics of these effects suggest that INPX stimulates the production of precursor cells and initiates a cascade of lymphocyte differentiation capable of producing long-term restoration of cell-mediated immunity. These data indicate that INPX may be beneficial to patients with PGL.

INTRODUCTION

Acquired immune deficiency syndrome (AIDS) was first described in 1981 and the number of reported cases has been increasing exponentially for the past several years (1). AIDS is most commonly observed in male homosexuals and is characterized by a pattern of cellular immunodeficiency that allows the development of opportunistic infections and Kaposi's sarcoma (2, 3). A milder syndrome, referred to as unexplained or persistent generalized lymphadenopathy (PGL) has been recognized in homosexual men (4, 5). This PGL syndrome can be a prodromal form of AIDS (6). Comparative studies suggest a continuum of immunodeficiency with its most severe manifestations in AIDS and relatively less immunologic abnormality in patients with PGL. Even asymptomatic homosexuals tend to be immunodeficient when compared to heterosexual controls or monogamous homosexual men (2, 7, 8).

The infectious agent responsible for AIDS has been tentatively identified as a human retrovirus, i.e., human T-cell lymphotropic virus type III (HTLV-III) or lymphadenopathy associated virus (LAV) (9, 10).

In patients with either AIDs or PGL, immunomodulation is indicated to correct the underlying defect in cell-mediated immunity. Here, we report the results of a double-blind placebo controlled clinical trial of the immunomodulating agent, inosine pranobex (INPX). INPX has been shown to be capable of *in vitro* en-

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hancement of mitogen-induced lymphocyte proliferation, macrophage activation, and T-rosette formation (11, 12). It has also shown clinical efficacy against various viral infections including influenza (13), genital herpes (14, 15), and subacute sclerosing panencephalitis (16). *In vitro* studies in lymphocytes from AIDS patients suggest that INPX normalizes production of interleukin 1 (IL-1) as well as both production of and binding sites for interleukin 2 (IL-2) (17, 18). Mitogen responses of AIDS patients are also increased by INPX *in vitro* (19, 20). A recent *in vivo* study suggested that the drug may be more effective in the prodromal stages of AIDS than in patients who have developed opportunistic infections or Kaposi's sarcoma (21). The present study examined the effects of INPX on a population of PGL patients who were considered to be at risk of developing AIDS.

MATERIALS AND METHODS

Patients

Patients were selected for the study if they (a) were male homosexuals or iv drug users at risk of developing AIDS, (b) had unexplained PGL, and (c) were immunodepressed as indicated by a T-helper/suppressor ratio less than 1.5, T-helper cell number less than 700/mm^3, or NK cell activity below the laboratory normal range (10% of specific lysis). Patients were excluded if they had opportunistic infections or Kaposi's sarcoma, were critically ill or were currently receiving steroids, cytotoxic immunosuppressive agents, radiotherapy, or other immunotherapy. A total of 74 patients gave their informed consent and were entered into the study.

Experimental Procedure

Upon admission to the study, patients were randomized to receive either (a) 6 tablets (3 mg/day) of INPX, (b) 2 tablets (1 g/day) of INPX, (c) 6 tablets/day of placebo, or (d) 2 tablets/day of placebo. Patients were treated for 28 days. Immunologic assays, hematology, and serum uric acid determinations were performed before treatment (Day 0) and on Days 7, 14, and 28, as well as 2 months (Day 90) 5 months (Day 180), and 11 months (Day 360) after the cessation of treatment. Complete physical and laboratory examinations including blood chemistries were performed on Days 0, 28, 180, and 360.

Immunologic Assays

Lymphocyte separation. Peripheral blood (50 ml) was taken by venipuncture in a plastic syringe containing 15 U/ml preservative-free heparin. Separation of peripheral blood lymphocytes (PBL) was achieved by Ficoll–Hypaque gradient centrifugation (22). Briefly, 50 ml of blood was diluted with an equal volume of saline overlaid onto a 25 ml Ficoll–Hypaque gradient, and centrifuged at 850*g* and 20°C for 45 min. The lymphocytes in the buffy coat interphase were washed twice in saline and resuspended in RPMI-1640 supplemented with 20% heat-inactivated autologous or heat-inactivated pooled AB plasma.

Surface Markers

Mononuclear cells were analyzed for cell surface phenotypes by indirect immunoflourescence. Monoclonal antibodies directed against E receptors of human T lymphocytes (T11), helper T lymphocytes (T4), and suppressor/cytotoxic T lymphocytes (T8) were obtained from Coulter Immunology, Inc. (Hialeah, Fla.). An aliquot containing 1×10^6 washed cells in 0.1 ml of medium was treated with 5 μl of unconjugated monoclonal antibody for 45 min on ice water in a 12 × 75-mm test tube and washed three times with PBS. Cells were then treated with 0.1 ml of a 1:40 dilution of goat anti-mouse IgG conjugated with flourescein isothiocyanate (Tago, Calif.), incubated on ice for 45 min, washed three times, and resuspended in 1 ml of 1% paraformaldehyde in PBS. At least 1×10^4 lymphocytes per sample were analyzed for fluorescence (excitation at 488 mm) in a Becton–Dickinson FACS analyzer. Volume gates were set on the lymphocyte peak and the number of lymphocytes stained with a given reagent was determined by subtraction of cells stained with an isotype-matched control antibody. Data were stored, displayed, and analyzed using a Hewlett–Packard computer (HP-85) and Consort 20 software.

NK assay. K562 cells, a myelogenous leukemia cell NK target-cell line (23) were maintained in optimal growth and passed twice weekly in RPMI-1640, pH 7.3, fortified with 10% heat-inactivated pooled human serum (GIBCO, Grand Island, N.Y.), 10 μg/ml gentamicin (Wyeth Laboratories, Philadelphia, Pa.), and 25 m*M* Hepes (Sigma Chemical Co., St. Louis, Mo.). K562 cells suspended in GlucoseGVB^{-+}, pH 7.3 (Cordis Laboratories, Miami, Fla.) at a concentration of $3–5 \times 10^6$ cells/ml were labeled for 50 min at 37°C with 150–200 μCi ^{51}Cr (Sodium Chromate, New England Nuclear, Boston, Mass.). The labeled cells were washed three times with RPMI-1640 (pH 7.3) supplemented with 10% heat-inactivated fetal calf serum. The cytotoxic assays were performed at an effector to target cell ratio of 50:1. NK cell-mediated cytotoxicity was studied in a total volume of 0.2 ml in u-bottom 96-well microtiter plates (Linbro Scientific, Hamden, Conn.). Fifty microliters of RPMI-1640 medium with 10% heat-inactivated FCS was first pipetted into each well, followed by 0.1 ml PBL, and finally 10^4 ^{51}Cr-K562 in 0.05 ml was added. The plates were incubated in a humidified atmosphere containing 5% CO_2 for 4 hr. The supernatants were harvested with Titerteck Supernatant Harvesters (Flow Laboratory, Rockville, Md.). The amount of radioactivity released into the medium was measured by counting the filters in a Gamma counter (Packard Auto-Gamma Spectrometer). All assays were performed in quadruplicate. Spontaneous release of ^{51}Cr (target cells incubated in the absence of effector cells) was usually about 6–7%. The maximum ^{51}Cr release was determined by incubated target cells with 3% cetrimide (Sigma Chemical Co., St. Louis, Mo.). The percentage of specific lysis was calculated according to the formula:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100.$$

Statistical Methods

At one, three, six, and twelve months (Days 28, 90, 180, and 360) after the start of treatment, changes in clinical status since the last examination were characterized as "improved," "slightly improved," "unchanged," "slightly worse," or "worse" based on current signs and symptoms as well as the frequency and severity of overt infection. Cumulative improvement scores were analyzed as a five-point scale using the non-parametric Mann–Whitney *U* Test with a correction for ties (24). In this analysis, each dosage level of INPX was individually compared to the combined (6 tablets/day and 2 tablets/day) placebo group and one-sided tests of statistical significance were employed.

Immune response variables were analyzed using Students' *t* test with each dosage level of INPX compared to the combined placebo group. After Day 0, the change in each value relative to Day 0 was used for these comparisons. One-sided tests of statistical significance were employed.

RESULTS

Patient Population

Of the 74 patients enrolled, 61 completed the 28-day drug regimen and are analyzed here. Of the 13 patients who did not complete the study, 7 were discontinued because they were found not to have been immunodepressed on Day 0 and 2 did not have lymphadenopathy. Only one patient who completed the 28 day course of drug treatment failed to return for the 6-month follow-up. The three groups were similar in terms of demographics and immune competence. Mean age ranged for 33 to 35 years while mean weights ranged from 151 to 155 lb.

Clinical Improvement

Figure 1 shows mean cumulative clinical improvement score at each examination. By Day 180, improvement had been observed in 11 of 21 patients (52%) in the high dose group and 5 of 21 patients (24%) in the low dose group as compared to 3 of 19 patients (16%) in the placebo group. Improvement was characterized by an increased sense of well-being and appetite and reduction of lymph node pain, night sweats, weight loss, skin rashes, and thrush-associated discomfort. Statistical analyses using the Mann–Whitney *U* test indicated significantly more improvement in the high dose group than in placebo at all time periods.

Immune Status

Table 1 presents the effects of INPX on NK cell activity, total T lymphocytes and T helper cells. Comparison of the two placebo groups (2 vs 6 tablets/day) indicated that the only significant effect was a larger increase in NK cell activity in the 6 tablet/day group on Day 180. These groups were, therefore, combined as a single placebo group. INPX, at a dose of 3 g/day for 28 days produced a significant increase in NK cell activity that, as compared to placebo, first reached statistical significance on Day 14 and was still elevated on Day 360, 11 months after the cessation of treatment. Significant increases in the number of T lymphocytes and T helper cells were observed on Day 90 while a significant increase in the number of T suppressor cells (data not shown) was observed on Day 28.

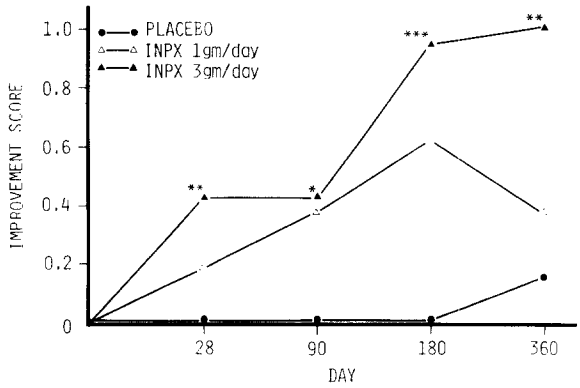

FIG. 1. Effect of inosine pranobex (INPX) on mean cumulative clinical improvement scores. On each examination, patients were scored as improved (+2), slightly improved (+1), no change or not examined (0), slightly worse (−1) or worse (−2). * = $P < .05$, ** = $P < .025$, *** = $P < .01$. Significantly more improved than the placebo group using one-sided Mann–Whitney U tests.

TABLE 1

EFFECT OF INOSINE PRANOBEX ON NATURAL KILLER (NK) CELL ACTIVITY(%), TOTAL T LYMPHOCYTES (T-11 CELLS/mm³), AND TOTAL T HELPER CELLS (T4 CELLS/mm³)

	Placebo			1 g/day			3 g/day		
Day	*N*	Mean	SD	*N*	Mean	SD	*N*	Mean	SD
NK									
0	19	11.3	(7.7)	21	16.5	(10.8)	21	11.9	(6.7)
7	19	14.6	(11.5)	20	17.4	(11.5)	21	19.3	(8.4)
14	19	13.4	(10.2)	20	19.3	(15.9)	21	26.6	(15.6)***
28	17	16.2	(14.2)	19	25.0	(13.5)	17	22.8	(14.8)
90	19	18.1	(11.6)	21	23.5	(10.4)	21	25.0	(10.3)*
180	18	13.6	(6.6)	20	18.1	(7.5)	20	22.3	(8.3)***
360	15	12.8	(4.4)	15	15.1	(6.1)	15	21.0	(5.8)***
T-11									
0	19	1288	(615)	21	1265	(826)	21	1210	(449)
7	19	1071	(376)	21	1143	(723)	21	1141	(341)
14	19	1060	(479)	21	1072	(586)	21	1186	(466)
28	19	1244	(611)	21	998	(569)	21	1391	(497)
90	19	1312	(539)	21	1287	(589)	21	1562	(531)**
180	18	1347	(577)	20	1232	(468)	19	1366	(453)
360	15	1204	(575)	15	1134	(448)	16	1026	(462)
T-4									
0	19	625	(281)	21	545	(341)	21	515	(161)
7	19	540	(248)	21	497	(305)	21	478	(177)
17	19	536	(223)	21	475	(206)	21	508	(188)
28	19	594	(310)	21	391	(187)	21	563	(170)
90	19	647	(321)	21	573	(268)	21	686	(292)*
180	18	595	(260)	20	532	(235)	19	572	(154)
360	15	492	(238)	15	452	(180)	16	488	(238)

* $P < .05$, **$P < .025$, ***$P < .01$ (significantly greater than the placebo group using one-sided t test comparisons of change from baseline).

Side Effects and Adverse Reactions

The only side effect reported was an expected transient increase in serum uric acid resulting from metabolism of INPX to uric acid via normal biochemical pathways (25). In the 3 g/day group, mean serum uric acid increased from 5.0 mg/dl to a peak of 6.6 mg/dl after 7 days of drug treatment. After 28 days of drug treatment, the mean level remained elevated (6.1 mg/dl) but by Day 90 (the first time serum uric acid levels were determined after cessation of treatment) it dropped to 5.0 mg/dl. In the 3 g/day group, there were a total of 6 elevated (>8.5 mg/dl) serum uric acid levels observed in 4 patients. The highest uric acid level observed was 9.9 mg/dl. A total of eight minor adverse reactions were reported in the three groups but only two of these (cases of nausea in the high dose group) were possibly drug related. No patients were discontinued from the study because of adverse reactions to INPX.

DISCUSSION

INPX, administered at a dose of 3 g/day for 23 days, restored a number of immune parameters in this group of immunodepressed PGL patients. The most striking effect of the drug was the substantial increase in NK cell activity which rose from a mean of 11.9 to 26.6% by Day 14 and was still significantly elevated by Day 180, 5 months after the cessation of treatment. Early potentiation of NK activity was not unexpected, having been observed *in vitro* (26, 27) and in animal models *in vivo* (28, 29). The subsequent increases in the numbers of T lymphocytes and T helper cells, although not of the same magnitude, indicate that INPX may trigger a chain of events that continues to affect the immune system long after drug treatment has been terminated.

The mechanism of INPX's effect on NK cell activity is not well understood although it is known that, under specific conditions, INPX can potentiate the activity of interferon (30, 31) and IL-2 (18, 32, 33), two known inducers of NK cell function. The time course of NK potentiation suggests that the drug may induce the production of undifferentiated stem cells rather than modulating the activity of existing NK cells in the affected patients. Within a relatively short period to time, these stem cells may assume the function of NK cytotoxicity. This would be expected since NK cells are relatively primitive and represent an early product of stem cell differentiation. As a consequence of INPX treatment, either the NK cells themselves or another lineage of stem cell may undergo further differentiation to yield cells that are phenotypically recognizable first as T lymphocytes (T11) an later as T helper cells (T4).

The time course of immunorestoration observed in this study is quite similar to that observed in immunodeficient patients treated by transplantation of bone marrow or fetal thymus (34). In these patients, human T-lymphocyte differentiation antigens (HTLA) appear within several weeks and the capacity to form E-rosettes is observed soon after their appearance. Within several months, the capacity to produce a proliferative response to allogeneic cells or mitogens such as concanavalin A (Con A) and PHA appears.

Clinically, it is clear that INPX, especially at a 3 g/day dose, was of benefit to

this PGL patient population. While this effect was first seen at the end of the treatment period (Day 28), it was even more pronounced at Day 180.

These data suggest that INPX can be an effective immunorestorative agent, counteracting the immunodeficiencies present in PGL patients who are, presumably, at risk of developing AIDS. The kinetics of the various potentiated immunologic responses suggest that the drug is capable of increasing NK cell activity and the number of cells expressing T11 and T helper (T4) antigens. However, it should be noted that the effects observed here are due to only one 28-day treatment cycle with INPX and do not constitute complete immunorestoration; these patients cannot be considered to be "cured." The results of this double-blind 28-day placebo-controlled trial suggest that INX may be a useful approach for the treatment of patients with PGL.

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