

Cell proliferation and natural killer cell activity by polyherbal formulation, Immu-21 in mice*

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Immunomodulatory activity of an Ayurvedic polyherbal formulation, Immu-21 containing extracts of *Ocimum sanctum*, *Withania somnifera*, *Embllica officinalis* and *Tinospora cordifolia* was studied on proliferative response of splenic leukocytes to T cell mitogens, concanavalin (Con)-A and phytohemagglutinin (PHA) and B cell mitogen, lipopolysaccharide (LPS) *in vitro* by [³H]-thymidine uptake assay in mice. The cytotoxic activity of Immu-21 was tested by measuring the splenic leukocyte natural killer (NK) cell activity against K 562 cells. Intraperitoneal (ip) treatment with Immu-21 (30 mg/kg) once a day for 14 and 21 days did not cause change in body weight and spleen weight, whereas splenocytes/spleen count was increased. Treatment of Immu-21 (30 mg/kg, ip) for 14 days and 1 mg/kg for 21 days significantly increased LPS induced leukocyte proliferation. NK cell activity was significantly increased when mice were pretreated with Immu-21 (10 and 30 mg/kg, ip) once a day for 7 days. The results indicate that pretreatment with Immu-21 selectively increased the proliferation of splenic leukocyte to B cell mitogen, LPS and cytotoxic activity against K 562 cells in mice.

Immunosuppression is the common adverse effect accompanying the treatment of chemotherapeutic agents^{1,2}. The immune system is known to be involved in the etiology and pathophysiological mechanisms of several diseases³. Inflammatory diseases of the skin, gut, respiratory tract, joints and central organs and infectious diseases are now primarily considered as immunological disorders and many neoplastic transformations appear due to immune suppression⁴. The function and efficiency of the immune system may be influenced by many exogenous and endogenous factors resulting in either immunosuppression or immunostimulation. Several agents have been shown to possess an activity to normalize or modulate pathophysiological processes and are called immunomodulatory agents⁵.

Immu-21, a polyherbal Ayurvedic formulation contains the extracts of *Ocimum sanctum*, *Withania somnifera*, *Embllica officinalis* and *Tinospora cordifolia*. The immunorestorative properties of Immu-21 have been reported in different mammalian systems. Cell-mediated immune response was demonstrated in Immu-21 treated rats administered with azathioprine⁶.

Immu-21 significantly enhanced the total RBC, WBC and macrophage counts in albino mice. It also significantly potentiated humoral immunity in rabbits and showed significant protection against UV rays, cyclophosphamide and cyclosporin A induced immunosuppression⁷.

Clinical evaluation indicates that it has no adverse effect and is quite safe for human use⁸. Immu-21 showed a very good adjuvant effect when administered together with ciprofloxacin in patients suffering from conjunctivitis and corneal ulcer⁹. In clinical study with confirmed AIDS (HIV I) patients, Immu-21 showed enhancement of CD4 and CD8 counts and the stimulation of cytokine production (through T helper cells) and control of viral multiplication¹⁰. However, the exact mechanism of immunorestoration of Immu-21 has not been tested in humans. The individual component of the above herbal formulation was reported to have immunomodulatory activity in different test systems¹¹⁻¹⁵. *Ocimum sanctum* has been reported to be a chemopreventive agent¹⁶. Its anti-inflammatory activity was reported in rat by Singh *et al.*¹⁷. It also possesses hypoglycemic effect in normal and diabetic rats¹⁸. *Withania somnifera* herbal tonic and to treat rheumatic pain, nervous disorders, hypertension and inflammation^{19,20}. It also exhibits anti-tumour and radiosensitizing effects in mice *in vivo*^{21,22}. *Tinospora cordifolia* is mainly used to

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treat dyspepsia, fever, urinary diseases and the bitter constituents present in it exhibit antispasmodic, antipyretic and anti-inflammatory properties²³.

Different agents of plant origins and their polyherbal formulation are reported to interact with immune system in a complex way and modulate the pathophysiological process²⁴⁻²⁶. Until recently, a major limitation to the use of immunostimulants has been that their effects are generalised throughout the immune system and not specific for a particular cell type or antibody²⁷. In order to reveal the immunopotentiating properties of Immu-21, investigations have been carried out on the lymphocyte proliferation with different mitogens *in vitro* and the natural killer cell activity against cancer cell line K 562. Therefore, in the present study, an effort has been made to evaluate the effect of pretreatment of animals with Immu-21 on proliferative response of splenic leukocytes to B cell mitogen, lipopolysaccharide (LPS) and T cell mitogens, concanavalin A (Con-A) and phytohemagglutinin (PHA). Further, the cytotoxic activity of splenic leukocytes of Immu-21 pretreated mice has been evaluated against K 562 erythroleukemic cell line.

Materials and Methods

Animals—Swiss male mice (Central Animal Facility, NIPER, India) weighing 20-25 g were housed 6 per cage in a room with controlled temperature ($22^{\circ}\pm 1^{\circ}\text{C}$), humidity ($50\pm 10\%$) and light (0600-1800 hrs). Standard laboratory animal feed purchased from Pranav Agro Industries, New Delhi was fed to the animals *ad libitum*.

Chemicals—Con-A, PHA, LPS, penicillin and streptomycin were obtained from Sigma, St. Louis, USA. Roswell Park Memorial Institute-1640 (RPMI-1640) and fetal bovine serum (FBS) were purchased from Himedia, Mumbai and fetal calf serum (FCS) from Gibco BRL, USA. [³H]-thymidine (specific activity 12 Ci/mmol) and sodium chromate [⁵¹Cr] (specific activity 35.5 mCi/mg) were obtained from Bhaba Atomic Research Centre, India.

Immu-21—The polyherbal formulation (Batch No. SU-01) used in the present investigation was supplied by Indian Herbs (Saharanpur, U.P., India), which contains equal proportions of individual components. Aqueous alcoholic extract of *T. cordifolia* was prepared from the stem and *W. somnifera* was from the root. Similarly, water-soluble extracts were prepared from freshly obtained fruits of *E. officinalis* leaves of *O. sanctum*. To identify and characterize

these active components of polyherbal formulation, HPTLC fingerprinting was done. The preparation of polyherbal formulation and the characterization of active components present in it were done by Indian Herbs. It was supplied in powder form. The formulation was dissolved in normal saline and stirred continuously for approximately 30 min. It was administered to the animals immediately after thorough shaking and was kept in the refrigerator for subsequent use.

Drug administration and tissue collection—Mice were randomly assigned in to different groups. The stock solution (3 mg/ml) was prepared from the powder. The animals were administered one of the three doses of either Immu-21 (1, 10 and 30 mg/kg) or the vehicle through ip injection. Normal saline was used as the vehicle and the volume of administration was 10 ml/kg to the animals. The mice were administered with Immu-21 once a day for 7 days for NK cell activity study whereas 14 and 21 days for mitogen stimulation assay. In NK cell activity study, vehicle treated group was administered cyclophosphamide (CP; 40 mg/kg, ip) 24 hrs before sacrifice. One hour after the administration of the last dose, mice were sacrificed by cervical dislocation and the spleen was removed aseptically. The splenocytes were prepared by modified method as described by Bhargava *et al*²⁸.

Each mouse's spleen was prepared as a single cell suspension by cutting the spleen capsule several times and gently expelling the contents through a cheese cloth in supplemented RPMI-1640 enriched with 10% fetal bovine serum. The suspension was centrifuged at 250 g for 5 min. The supernatant was discarded and the pellet was resuspended in supplemented RPMI-1640 enriched with 10% FBS. Each mouse's splenic leukocytes were counted using a Neubauer's counting chamber and the cell viability was determined by trypan blue dye exclusion method. The cell suspensions were adjusted to a concentration of 5×10^6 leukocytes/ml in supplemented RPMI-1640 enriched with 10% fetal bovine serum.

Mitogen stimulation assay—Con-A, PHA and LPS were prepared to a concentrations of 50 and 500 µg/ml and 10 µl of each of the mitogens were added in triplicate into the wells of 24-well flat bottom microtiter plate (Laxbro, India). The final concentration was adjusted to 5×10^6 leukocytes/ml with supplemented RPMI-1640 containing 10 % FBS and 200 µl of the cell suspension was added into each well of the microtiter plates. The total volume was made

up to 450 μ l by adding supplemented RPMI-1640. The cells were cultured in the microtiter plate in CO₂ (5%) incubation at 37°C temperature. Each well of the cultures was pulsed with 0.05 μ Ci of [³H]-thymidine in 50 μ l supplemented RPMI-1640 during the last 5 hr of a 48 hr incubation period to make the final mitogen concentrations of 1 and 10 μ g/ml. The cells were separated by centrifugation (10,000g for 30 min) and the incorporation was determined with a liquid scintillation counter (Wallac 1409; 49% efficiency).

NK cell activity—The NK cell activity was measured by a modified method of Talmadge²⁹. In the present investigation, the natural killer cell activity was detected using the highly sensitive K 562 tumor cell line as described by Pross and Maroun³⁰. Target cell line (K 562) was maintained in complete medium (RPMI-1640, Himedia, Mumbai) with excess L-glutamine (2 mM), penicillin (50 μ g/ml), streptomycin (50 μ g/ml) and 10% FCS. On the day of the experiment, the target cells were radiolabelled with 200 μ Ci of sodium chromate [⁵¹Cr] for 70 min. The target cells were washed thrice in supplemented RPMI - 1640 to remove any exogenous ⁵¹Cr. The labelled target cells were diluted in supplemented RPMI-1640 and splenic lymphocytes were mixed with a constant number of ⁵¹Cr - labelled target cells in V-bottom microplates (Laxbro). The microtiter plates containing the cell mixture were incubated at 37°C with 5% CO₂ for 16 hr. Immediately before the harvest, the target cells in one set of control wells were treated with 10% trichloroacetic acid for lysis of cells and to determine the maximum possible release of ⁵¹Cr. The cells were separated by centrifugation (150 g for 10 min) and 100 μ l aliquots were removed for counting in gamma

counter. The percentage of cell mediated lysis (CML) was calculated as follows:

$$\text{CML (\%)} = \left[\frac{\text{cpm (test)} - \text{cpm (medium)}}{\text{cpm (max)} - \text{cpm (control)}} \right] \times 100$$

Statistical analysis—The results were expressed as mean \pm SE. The data were subjected to either one-way analysis of variance (ANOVA) followed by multiple group comparison test Scheffe's S-test or one-tail Students 't' test.

Results

Effect of Immu-21 on body weight, spleen weight and cellularity—Pretreatment of Immu-21 for 14 and 21 days did not cause any changes in the body weight (Table 1). Although, there was no change in spleen weight in different groups, significant increase in the number of splenocytes per spleen was observed on pretreatment with Immu-21 (30 mg/kg, ip) for 14 and 21 days (Table 1).

Mitogen stimulation assay—Administration of Immu-21 for 14 and 21 days did not cause any changes in leukocyte proliferation to Con-A and PHA (Fig. 1). However, significant effect was seen on LPS at a dose of 30 mg/kg (14 days) and 1 mg/kg for 21 days ($P < 0.05$). The increase in mitogen induced lymphocyte proliferation was around 6 folds with Immu-21 (30 mg/kg) for 14 days. Interestingly, no difference in mitogen induced cell proliferation was observed in the mice treated with 10 and 30 mg/kg of Immu-21 for 21 days.

NK cell activity—The effect of pretreatment of Immu-21 (1, 10 and 30 mg/kg, ip) for 7 days on the NK cell activity against K 562 cell line is shown in Table 2. The results of the study were expressed as percentage change in lytic activity over the control.

Table 1—Body and spleen weights of mice pretreated with immu-21 for 14 and 21 days
[Values are mean \pm SE from 5 animals in each group]

Weight and Cellularity	Dose of Immu-21 (mg/kg)			
	Control	1	10	30
14 Days treatment				
Body Weight (g)	23.8 \pm 0.9	24.0 \pm 0.9	23.8 \pm 0.3	24.0 \pm 0.4
Spleen Weight (mg)	71.8 \pm 5.0	77.8 \pm 6.1	102.8 \pm 13.4	100.0 \pm 6.3
Cells / spleen (X 10 ⁶)	48.1 \pm 1.7	54.0 \pm 4.0	57.8 \pm 7.0	72.3 \pm 5.8*
21 Days treatment				
Body weight(g)	25.2 \pm 0.5	22.5 \pm 0.6	24.0 \pm 0.5	23.8 \pm 0.9
Spleen Weight (mg)	106.8 \pm 12.2	70.2 \pm 3.3	99.8 \pm 8.7	104.2 \pm 10.9
Cells / spleen (X 10 ⁶)	49.5 \pm 3.1	48.0 \pm 1.8	65.5 \pm 5.2	74.2 \pm 7.5*

* $P < 0.05$ significantly differed from respective control group

Cyclophosphamide (40 mg/kg, ip) treatment resulted in significant decrease in the cytotoxic activity of splenic leukocytes. Greater degree of NK cell activity was observed with pretreatment of Immu-21 at the dose of 10 and 30 mg/kg ($P < 0.05$).

Discussion

The results of the present study indicate that Immu-21 has immunorestorative and cytotoxic activity in mice *in vivo*. Immu-21 (1, 10 and 30 mg/kg, ip), when given for 14 and 21 days did not affect the body weight and spleen weight, but did increase the cellularity of splenic leukocytes indicating the growth promoting effects of Immu-21. *Withania somnifera* has been reported to significantly increase the white blood cell count and prevented myelosuppression against immunosuppressive drugs in mice *in vivo*³¹.

The immune system is composed of two major mechanisms: cell-mediated and humoral (antibody) immunity³². T and B lymphocytes are generally con-

sidered the major effector functions associated with cell mediated immunity. The immunostimulating agents can produce an effect through either cellular or humoral immunity or both. The measurement of lymphocyte proliferation using T cell mitogens, Con-A, PHA and B cell mitogen, LPS, enables the detection of immunomodulation with specific activity towards T and B lymphocytes, respectively³³. Immu-21 (1, 10 and 30 mg/kg, ip for 14 and 21 days) did not alter the

Table 2—Effect of pretreatment of Immu-21 for seven days on NK cell activity in mice.

[Values are mean \pm SE from 6 animals]

Pretreatment	Change in lytic activity (%)
Normal saline	100
Normal saline + CP (40 mg/kg)	80.4 \pm 2.4*
Immu-21 (1 mg/kg)	110.0 \pm 6.5
10 mg/kg	127.8 \pm 8.8*
30 mg/kg	122.2 \pm 4.6*

* $P < 0.05$ vs normal saline treated group

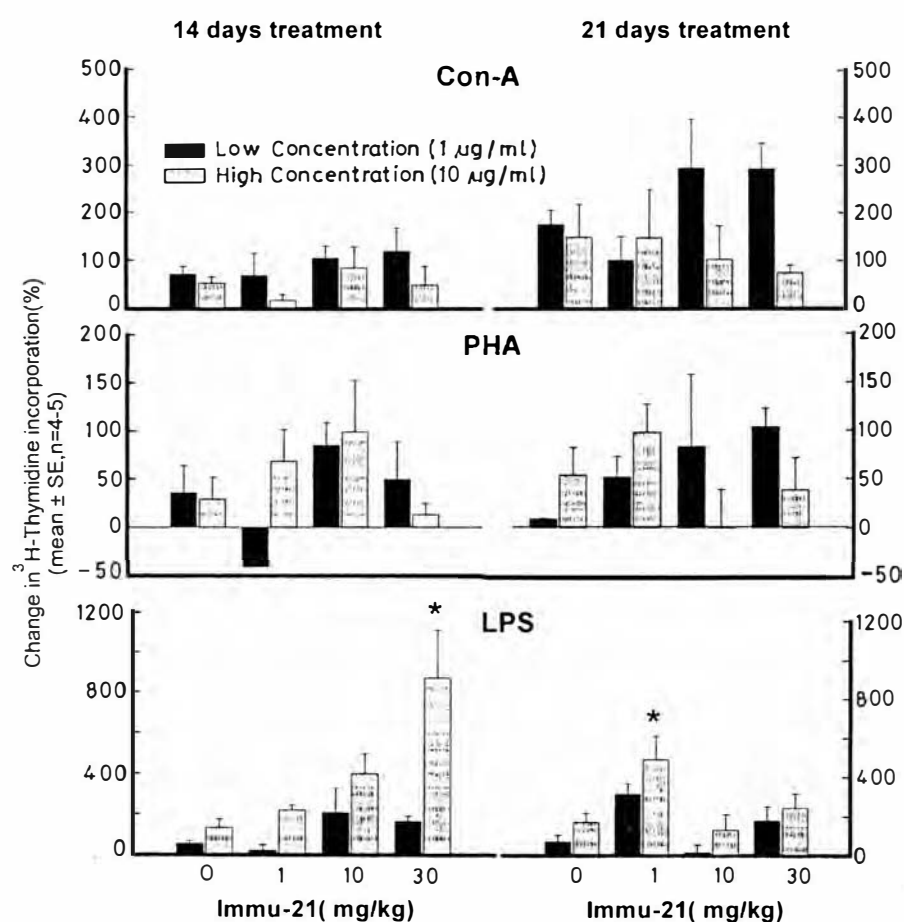


Fig. 1—Effect of Immu-21 (1, 10 and 30 mg/kg, ip) for 14 and 21 days on mice splenic leukocyte proliferation by mitogens. (Con-A=concanavalin; PHA= phytohemagglutinin; LPS=lipopolysaccharide) $P < 0.05$ vs control group

proliferation of splenic leukocytes to T cell mitogens, Con-A and PHA (1 and 10 µg/ml) as no significant change in the incorporation of [³H]-thymidine was observed (Fig. 1). It has already been reported that incorporation of tritiated thymidine into nascent DNA provides a highly sensitive means of assessing cell proliferation and the quantitative assessment of compound effects on the rate of cellular DNA synthesis^{34,35}. The proliferation of splenic leukocytes to B cell mitogen, LPS (10 µg/ml) was increased in the group which was pretreated with Immu-21 (30 mg/kg, ip) for 14 days and with 1 mg/kg for 21 days. However, lower doses (1, 10 mg/kg, ip) did not alter the thymidine incorporation. Higher doses of Immu-21 (10, 30 mg/kg, ip) treated for 21 days did not alter the thymidine incorporation. This observation is possible either due to the toxic effect of Immu-21 at higher doses or due to the bell shaped dose response curve of Immu-21 on prolonged pretreatment. Wong and Tan³⁶ observed similar effect of suppression of *in vitro* splenic leukocyte proliferation activity with a plant extract at higher doses in the presence of mitogens (Con-A and LPS) with normal animals. However, no specific claim can be done from the present study on the exact mechanism of inhibition of proliferation at higher dose (10 mg/kg) of Immu-21 in mice *in vivo*.

NK cells are a population of lymphocytes unrelated to either T cells or B cells and are able to non-specifically destroy certain target cells and are thought to play an important role in nonspecific host resistance³⁷. These lymphoid cells are lacking of both T- and B- cells markers and has a great role to play in reducing the development of cancer and its metastasis³⁸. In the present experiment, CP was used as positive control and the NK cell activity was significantly inhibited in animals treated with CP 24 hr before the experiment. In a similar type of experiment by Xu *et al.*³⁹, CP was used for inhibition of NK cell activity to evaluate the immunopotentiating property of herbal compound. The present study indicates that pretreatment of animals with intraperitoneal injection of Immu-21 (10 and 30 mg/kg, ip for 7 days) significantly augmented the NK cell activity against K 562 cell line as measured by lytic activity. Similar results with another polyherbal compound, septilin (100 mg/animal/dose; five doses) administered through oral route was found to enhance NK cell mediated cytotoxicity both in normal as well as tumour bearing mice⁴⁰. The possible explanation for increase in NK cell activity could either be the result of direct activa-

tion of mature NK cells or could be the activation of precursor cells not previously capable of mediating cytotoxicity. Certain cytokines including IL-2 and interferon-gamma are capable of enhancing the cytotoxic potential of NK cells^{29,41}. Hence, estimation of different cytokines may elucidate the exact mechanism of action of this polyherbal drug in mice *in vivo*.

To summarise, the results of the study indicate that Immu-21 at appropriate dose can produce immunomodulatory effect and is capable of enhancing the cytotoxic potential of NK cells in mice *in vivo*. The findings further suggest that Immu-21 is a potential formulation which can restore immunosuppression associated with the use of chemotherapeutic agents. However, further studies are needed to elucidate the exact mechanism of action of the active components present in the polyherbal formulation involved in the stimulation of the immune system.

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