Research Paper

Effect of *Hemidesmus indicus* (Anantmool) extract on IgG production and adenosine deaminase activity of human lymphocytes *in vitro*

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ABSTRACT

Objective: To investigate the effect of *Hemidesmus indicus* extract on activities of human peripheral blood lymphocytes *in vitro*.

Materials and Methods: The total extract of the raw herb was obtained by methanol: isopropyl alcohol: acetone extraction, and used at different concentrations. Human peripheral blood lymphocytes (PBLs) were isolated, stimulated to proliferate using phytohaemagglutinin (PHA) or lipopolysaccharide (LPS), with and without different concentration of herbal extracts. Adenosine deaminase (ADA) activity and immunoglobulin (IgG) secretion from cultured PBLs were studied with the herbal extracts and appropriate controls.

Results: *Hemidesmus indicus* extract stimulated the cell proliferation at 1 mg/ml concentration significantly, after 72 h in culture. Viability of extract-treated PBLs was also maintained after culture. The extract increased the IgG production from cultured PBLs, when used at 1 mg/ml concentration. It also increased the ADA activity of PBLs after 72 h in culture.

Conclusion: An immunomodulatory activity of *H.indicus,* related to IgG secretion and ADA activity, is revealed during the study. The herbal extract has shown to promote the release of IgG by lymphocytes, and also the ADA activity after 72 h of culture.

KEY WORDS: Adenosine deaminase activity, immunoglobulin, sariva.

Introduction

Herbal preparations are effectively and extensively used for their medicinal properties, and have become increasingly popular worldwide.[1,2] Herbal medicines generally have fewer side effects than synthetic compounds, and their effectiveness can be improved by modern pharmacological methods.[3] Several plants used in the traditional system of medicines, have been shown to modulate immune response.[4-6] *Hemidesmus indicus,* commonly known as Anantmool or Sariva, belongs to family Asclepiadaceae and is a well-known drug in the ayurveda system of medicine. Various workers have studied the effector mechanism of *H. indicus* extract using different models.[7,8] Extract of this plant is reported to possess antiinflammatory, antipyretic, antioxidant and antiulcerogenic properties.[8-10] *H. indicus* extract is also found to inhibit lipid peroxidation and scavenge hydroxide radicals *in vitro*.[6] However, no studies have been reported on the effect of *H. indicus* extract on the immunoglobulin G (IgG) production and adenosine deaminase (ADA) activity of human peripheral blood lymphocytes (PBLs), *in vitro*. In the present study, we investigated the effect of *H. indicus* extract on the secretion of IgG and ADA activity, from unstimulated and lipopolysaccharide (LPS) or phytoheameagglutinin (PHA) stimulated human PBLs, isolated from the blood of consenting healthy volunteers. LPS and PHA stimulate specifically, B cells and T cells, respectively.[11-13] Immunoglobulins are secreted from B cells (plasma cells), and are responsible for humoral immunity. ADA is an important enzyme, whose physiological activity is related to lymphocyte proliferation and differentiation. The enzyme activity is significantly elevated in T cells, as compared to its activity in B cells.[14] The above parameters thus explored the effect of extract on humoral and cellular immunity.

Materials and Methods

Preparation of extract

The commercially available total extract of *H. indicus* was purchased from Innocon Foods (Pune, India), (Batch no. C/6476). The multiple solvent (methanol: isopropyl alcohol: acetone) extraction procedure, was used to prepare the extract by the supplier. Ten mili grams of the extract was suspended in 1 ml RPMI-1640 medium, (GibcoBRL). The suspension was

shaken vigorously on a vortex mixer. The insoluble components were quickly pelleted, and the remaining suspension was filtered twice through a 0.22-micron syringe filter. This filtrate was used as a stock (approximately 10 mg/ml), from which dilutions were made for incubation with PBLs. The stock solution was diluted in the medium before treatment with PBLs.

Isolation of peripheral blood mononuclear cells

Thirty consenting volunteers ranging in age from 16 to 45 years, participated in this study. The study was approved by Institutional Ethics Committee of Central India Institute of Medical sciences. Five mili litres of peripheral blood was collected into heparin-containing bottles and mixed in the ratio of 1:1 with RPMI-1640 medium, containing 10% heat inactivated newborn calf serum (NCS) supplemented with antibiotic-antimycotic solution (10,000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B per ml in 0.9% NaCl), (Himedia, Mumbai, India). The diluted blood was then layered over Histopaque1077 gradient (Sigma, St. Louis, M.O.), and centrifuged at 400 g for 40 min. Peripheral blood lymphocytes (PBLs) were harvested from the interphase layer, and washed twice with RPMI-1640 containing 10% NCS, and antibiotic-antimycotic solution. The PBLs concentration was adjusted to 2 x 10^6 viable cells/ml. Viability was determined by Trypan blue exclusion, and was consistently greater than 96%. All data were verified by atleast three separate experiments.

Culture of PBLs with extract

100 μl of 2 x 10^6 cells/ml (i.e. 2 x 10^5 PBLs) were cultured in 96 well microtitre plates, with or without addition of PHA (10 μl/ml, Gibco-BRL) or LPS (10 μg/ml, Sigma, St. Louis, M.O.). PBLs were also treated with 10, 100, 500 and 1000 μg/ml *H. indicus* extract. All the above cell treatments were performed in triplicate. After incubation at 37°C in 5% carbon dioxide for 72 h, cells were harvested for estimation of endpoints.

Cell proliferation assay

PBLs were stimulated with each of the four concentration (10, 100, 500 and 1000 μg/ml) of *H. indicus* extract. After 72h of incubation, 20 μl of XTT (2,3-bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-Tetrazolim-6-carboxanilide inner salt) (1 mg/ml)(Sigma, St. Louis, M.O.) containing Phenazine methosulfate (PNS) (0.92 mg/ml XTT) (Sigma, St. Louis, M.O.) was added to the wells, and then the plates were read after 4 h, at a test wavelength of 450 nm. Mean background values were obtained by scanning blank wells containing medium only.

Viability assay

Viability of PBLs was determined by the standard Trypan blue dye exclusion test. Approximately, 2 x 10^6 cells/ml of PBLs were treated with two fold diluted concentration of *H. indicus* extracts, and the control with medium alone, for 3 days in a humidified incubator at 37°C with 5% CO_2. The total number of viable and non-viable cells was counted under a microscope with the help of a haemocytometer, following staining by Trypan blue. The percentage of viable cells was calculated.

Assay of IgG production by ELISA

Enzyme linked Immunosorbent assay (ELISA) procedure[15] was used for the detection of human IgG from the culture supernatant, with a slight modification. Briefly, the protocol was developed by using standard human IgG (Bangalore Genei, Bangalore, India) and rabbit anti human IgG (Bangalore Genei, Bangalore, India), with different dilutions and TMB/H_2O_2 (tetramethyl benzidine/hydrogen peroxide) as substrate, till the color developed. For estimation of IgG, PBLs were harvested after 72 h of culture and centrifuged at 500 x g, to obtain cell free supernatant. 100 μl of culture supernatants were then coated on flat-bottom 96-well plates, for 3 h at 37°C. The wells were then washed thrice with phosphate buffered saline (PBS), pH 7.4, and then non-specific sites were blocked by adding 100 μl of PBS containing 0.5% bovine serum albumin (BSA), at 37°C for 1 h. After blocking, wells were washed with PBS, followed by addition of 100 μl of affinity-purified horseradish peroxidase-conjugated rabbit antihuman IgG (1:10,000 diluted in PBS) (Genei, Banglore, India), for 1 h at 37°C. After 1 h of incubation, wells were again washed with five changes of PBS. 100 μl of TMB/H_2O_2 substrate solution was added into the wells and incubated at room temperature, for about 15 min. The reaction was stopped with 100 μl of 2.5 N sulphuric acid, and the optical density was measured at 450 nm. Results are expressed in units/ml.

Adenosine deaminase assay

The intracellular ADA activity was studied after 72 h of PBL culture. Cells were harvested from 96 well plates, and collected by centrifugation. Cells were washed twice with ice-cold PBS, and resuspended in 0.1 ml of PBS. Cell lysates were prepared by 6 cycles of freezing at -80°C, and thawing at room temperature. The extracts were finally centrifuged at 10,000g for 5 min at 4°C, and supernatants were analyzed for enzyme activities and results were expressed as units/liter/min. A colorimetric assay was used for the measurement of ADA activity.[16] ADA activities were determined at 37°C, according to the method of Giusti and Galanti (1984),[17] based on the Bertholet reaction, that is, the formation of colored Indophenol complex from ammonia liberated from adenosine, and quantified spectrophotometrically at 640 nm.

Statistical analysis

Each experiment with PBLs was run in triplicate, and the results are expressed as their mean±SEM. The data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparisons test. $P<0.05$ was considered significant.

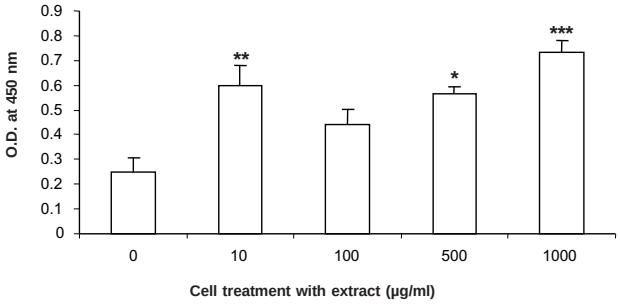
Results

Effect of extract on proliferation of PBLs

To estimate the mitogenic activity of *H. indicus* extract in PBLs, 2 x 10^5 cells were added to 96-well micro titreplates, and the cells were then stimulated with 10, 100, 500 and 1000 μg/ml of *H. indicus* extract. At the end of 72 h treatment incubation, the proliferation indexes were 140%, 77%, 128% and 195% of control, respectively. [Figure1]

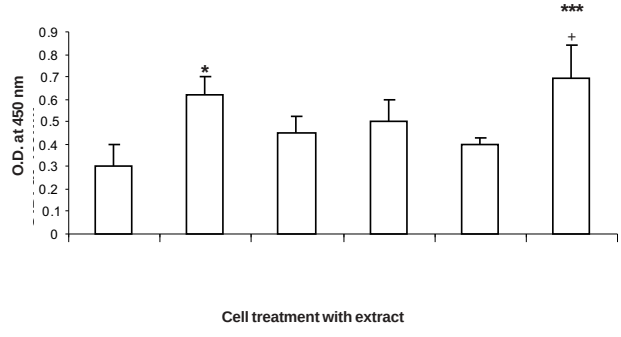
After simultaneous treatment with 1000 μg/ml *H. indicus* extract and LPS (10 μg/ml), the cell proliferation was significantly increased to 179% ($P<0.001$), whereas with LPS alone, the proliferation was 60%. In the case of simultaneous treatment of cells with PHA and 1000 μg/ml *H. indicus* extract, cell proliferation also increased to 102% and with PHA alone, the proliferation was 81% [Figure 2], but significance was not observed statistically.

Figure 1. Cell proliferation after 72 h with various concentration (10, 100, 500 and 1000 µg/ml) of *H.indicus* extract as measured by XTT assay.



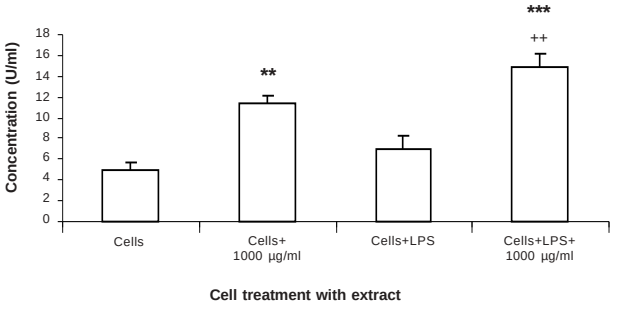
Significantly different from cells (0) control: *P<0.05, **P<0.01, ***P<0.001

Figure 2. Cell proliferation after incubation with 1000 µg/ml *H.indicus* extract and PHA (10 µl/ml) or LPS (10 µg/ml) as measured by XTT assay.



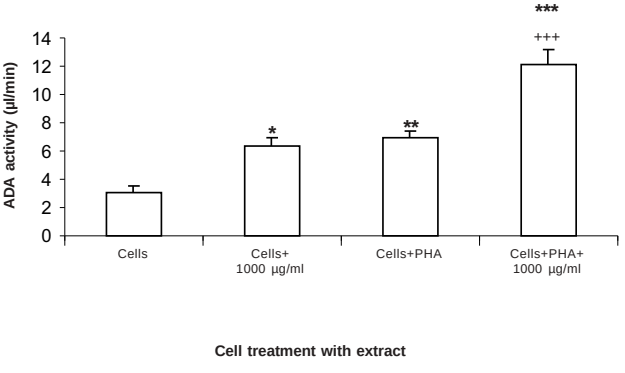
Significantly different from cells control: *P<0.05, ***P<0.001. Significantly different from LPS control: ^{+}P<0.005

Figure 3. IgG production by LPS treated and untreated PBLs after a 72 h incubation with 1000 µg/ml *H.indicus* extract.



Significantly different from cells control: ***P<0.001. Significantly different from LPS control: ^{++}P<0.01

Figure 4. ADA activity of PHA treated and untreated PBLs after a 72 h incubation with 1000 µg/ml *H.indicus* extract.



Significantly different from cells control: *P<0.05, **P<0.01, ***P<0.001. Significantly different from PHA control: ^{+++}P<0.001

Effect of extract on cell viability

Viability of cells was maintained after being treated with extract for 3 days, as compared to control cells without extract. This indicates that the *H. indicus* extract had no cytotoxicity, because the viability of stimulated cells was not significantly reduced after treatment with the extract for three days, as compared with controlled cells in medium alone (94.2±3.8% versus 95±2.6%)

Effect of extract on IgG production

In the absence of LPS, a 72 h incubation of PBLs with *H. indicus* extract at 1000 µg/ml concentration, significantly increased the production of IgG from mean of 5.05 units in the control, to a mean of 11.36 units (P<0.001). This represented an average change of 124%. [Figure 3] No significant changes compared with control, were observed at any of the lower concentration of *H. indicus* extract (10, 100 and 500 µg/ml) (data not shown). Incubation of PBLs with LPS (10 µg/ml) in combination with 1000 µg/ml of *H. indicus,* resulted in an significant increase in IgG production to a mean of 14.96 units, from a mean of 7.04 units in the LPS control (P<0.01), which corresponded to an average change of 107%. The other concentrations of extract had no detectable effect of IgG production, compared to the results obtained with LPS alone.

Effect of extract on ADA activity

In the absence of PHA, very less or no ADA activity was found in the cell lysates of PBLs culture for 72 h, with lower concentration of extract (10, 100, 500 µg/ml) (Data not shown). Therefore, under experimental conditions, extract alone did not enhance the ADA activity significantly at lower concentrations. Incubation of PBLs with 1000 µg/ml *H. indicus* extract showed a significant increase in ADA activity from a mean of 3.07 U/l/min in the control, to a mean of 6.3 U/l/min (P<0.05). This corresponded to an average change of 105%. Incubation of PBLs with PHA at optimized concentration (10 µl/ml) for 72 h resulted in a significant increase in ADA activity to a mean of 12.09 U/l/min, as compared to a mean of 6.9 U/l/min in the PHA control (P<0.001), which corresponded to an average change of 83%. [Figure 4]

Discussion

Many researchers have found *in vitro* effect of different herbal extracts on lymphocytes.[5,18,19] However, there is no study done on effect of *H. indicus* extract on human PBLs. The presented results demonstrated that, *H. indicus* extract significantly enhanced the cell proliferation in unstimulated human PBLs, highest at 1000 μg/ml. In the presence of LPS or PHA, *H. indicus* extract (1000 μg/ml) synergistically increased cell proliferation, compared with LPS or PHA alone. Therefore, the *H. indicus* extract might be acting as a mitogen for both B and T cells. We have also checked the effect of *H. indicus* on viability of these cells, and observed that the extract has no adverse effect on cell viability when exposed to 72 hours. B cell responses are usually measured by assays of secreted antibodies (IgG, IgM etc.). We demonstrated that the extract (1000 μg/ml) significantly enhanced the IgG secretion from PBLs, in absence or presence of LPS. LPS, which is known to be one of T-cell-independent (TI) antigen, activates B cells and differentiates B cells into IgG secreting cells.[11,12] *H. indicus* extract (1000 μg/ml), when added to LPS-stimulated, as well as unstimulated human PBLs, enhanced the IgG secretion after 72 h in culture. This indicates that extract alone also possess some mitogenic activity, which enhances the IgG secretion from PBLs, similar to LPS alone. Some of the components isolated from *H. indicus* are ketone, saponins, tannins, sterols, ß-sitosterol, stigmasterol, and sarsapic acid. One of these components might add to the mitogenic effect of lipopolysaccharide, which in turn might stimulate the secretion of IgG from PBLs. The existing data also indicates that this extract also stimulates the ADA activity from human PBLs. ADA is an enzyme capable of catalyzing the catabolism of purine bases (adenosine), and whose principal biologic activity is detected in T-lymphocytes.[20] The main physiologic activity of ADA is related to lymphocyte proliferation and differentiation, and its activity is closely related to T cell activation.[21] The results of ADA activity assay, showed that *H. indicus* extract significantly increased the ADA activity of unstimulated cells as well as PHA-stimulated cells, as compared to PBLs alone. PHA is a mitogen for T lymphocytes.[13] It binds to N-acetylgalactosamine glycoproteins expressed on the surface of T cells, which then activates the cells to proliferate. Thus T cells were major proliferating cells in PBLs activated with PHA. Therefore we can say that increase in ADA activity in PHA- stimulated PBLs treated with extract, might be due to T cell proliferation or activation, as the activity of ADA enzyme is subject to changes depending upon the degree of activity of the cell i.e., whether differentiation or activation occurs.[22] Therefore, we can conclude from the data obtained in our study, that the total extract of *H. indicus* has a stimulatory effect in terms of IgG production and ADA activity of human peripheral blood lymphocytes, *in vitro*.

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