

Immunostimulatory action of AC II — An ayurvedic formulation useful in HIV

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Received 29 May 2007; revised 28 September 2007

Immunostimulatory activity of AC II, a registered ayurvedic preparation prepared at Amala Ayurvedic Research Centre for treating HIV and AIDS is reported. AC II administration could significantly enhance the mitogen-induced proliferation of lymphocytes of spleen cells. It was also found to increase cell-mediated immune responses in normal and tumor-bearing control animals. Oral administration of AC II significantly enhanced Natural Killer cell activity in normal and tumor-bearing animals on the 7th day, which was observed earlier than the tumor-bearing control animals and normal animals. Antibody dependent cellular cytotoxicity (ADCC) was also increased in AC II treated normal and tumor-bearing animals. An early enhancement of antibody-dependent complement-mediated cytotoxicity was also observed by the administration of AC II in normal as well as tumor-bearing animals. Treatment with AC II elevated the levels of IL-2, TNF- α and IFN- γ in normal mice. Administration of AC II was also found to increase the cytotoxic T lymphocyte production in EL4 treated mice. These studies support the use of this immune stimulatory preparation in HIV patients.

Keywords: Cell mediated immunity, Cytotoxic T lymphocytes, Herbal drugs, Humoral immunity, Traditional medicine.

Acquired immunodeficiency syndrome (AIDS) continues to grow in pandemic proportions around the world. Etiologic agent is now has been widely established as Human Immunodeficiency Virus (HIV), which causes the profound depletion of CD4⁺ lymphocytes and there by produce immuno-suppression¹. The severity of the problem has launched a vigorous search for antiretroviral drugs against HIV. Despite the beneficial effects of currently available antiretroviral drugs in improving the quality of life of HIV/AIDS patients, the development of viral resistance, drug toxicity, high cost, unavailability and above all the lack of curative effect are the major shortcomings². As a result, the search for anti-HIV agents continues and much attention has been focused on natural sources particularly plant species³.

Herbal preparations because of their low preparation cost and nontoxicity could be administered for long periods of time in HIV seropositives. AC II, a registered Ayurvedic preparation was formulated at Amala Ayurvedic Research Centre to be given to HIV

patients. This preparation contains plant materials with known immunostimulating activity such as *Withania somnifera* (rhizome), *Syzygium aromaticum* (flower buds), *Mesua ferrea* (flower buds), *Elettaria cardamomum* (seeds), *Piper nigrum* (seeds), *Piper longum* (seeds) and *Zingiber officinale* (rhizome)⁴. AC II was prepared by taking a proportionate quantity of each of these ingredients, which were dried and powdered together. Using these preparations the Centre has been treating HIV seropositive individuals (symptomatic and nonsymptomatic patients) for the last 10 years. It has been observed that in symptomatic patients, administration of the drugs relieves the symptoms of these patients and in unsymptomatic patients the drugs delays the onset of symptoms⁵.

AC II can reduce the immunosuppression produced by irradiation and could increase the antibody titre and antibody forming cells in normal mice⁶. In the present study the cell-mediated immune responses of AC II has been analysed in normal and tumour bearing animals.

Materials and Methods

Cell lines — K562 cells were obtained from the National Centre for Cell Sciences, Pune. Ehrlich

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ascites carcinoma (EAC) cells were originally obtained from Cancer Institute, Adayar and maintained in Swiss Albino mice at Amala Cancer Research Centre. Sheep red blood cells were freshly collected in Alsevier's solution from slaughterhouse and washed thrice in normal saline.

Chemicals — RPMI and DMEM were purchased from Hi-media Laboratories, Mumbai. FCS was obtained from Kibbutz beit, Israel. Concanavalin-A was purchased from Sigma chemicals, St. Louis, MO, USA and phytohemagglutinin (PHA) from Difco Laboratories, USA. PPO and POPOP were purchased from SISCO Research Lab, Mumbai. ^3H -thymidine (sp. activity 10,000-25,000 mCi/mmol) was purchased from BARC, Mumbai, India. All other chemicals used in the experiments were analytical reagent grade and purchased locally.

Animals — BALB/c mice were obtained from the animal house of Amala Cancer Research Centre, Thrissur. They were housed in ventilated cages and given mouse chow (Sai Durga Foods and Feeds, Bangalore) and water *ad libitum*. All animal experiments were conducted after getting sanction from Institutional Animal Ethics Committee and as per the instructions of the Committee for the Purpose of Content and Supervisions of Experiments on animals (CPCSEA), Ministry of Environment and Forest, Government of India.

Preparation of extract — AC II, was supplied by Amala Ayurvedic Research Centre. Each time 10g of AC II was boiled in 500 ml distilled water for 1 hr. Filtrate was evaporated to dryness under vacuum at 50°C to 55°C using a rotary evaporator under reduced pressure. The yield of the preparation of AC II was 3.5 g. Evaporated extracts were reconstituted in distilled water.

Determination of effect of AC II on lymphocyte proliferation — Mice were divided into two groups (3 animals/group). Group I acted as untreated controls. Group II animals were fed orally with AC II (1g/kg body weight) every day for 5 consecutive days. The animals were sacrificed 24hr after drug treatment and spleen cells were used as a source for lymphocytes. Spleen cells (in triplicate) were incubated with PHA (2.5 µg/ml) and Con A (10µg/ml) at 37°C for 48hr. ^3H -thymidine (1µCi) was added to the cells 16hr prior and the cells were incubated for 72 hr. After incubation, 100µl medium was removed and 100µl of 6N NaOH (6N) was added to each well and further incubated at 37°C for 2 hr. The amount of radioactive

thymidine incorporated to DNA was counted using liquid scintillation counter.

Determination of cytokines — Mice were divided into two groups (3 animals/group). Group I was kept as normal. Group II animals were fed with 5 doses of AC II (1g/kg body weight orally). After 5 days, blood was collected, serum was separated and cytokine levels of IL-2, IFN-γ and TNF-α were determined by ELISA method using kits supplied by Endogen, USA.

Determination of NK cell activity, antibody dependant cellular cytotoxicity (ADCC) and antibody dependant complement mediated cytotoxicity (ACC) — Four groups of animals (12 animals/group) were used for the experiment. Group I animals were kept as normal. Group II animals received Ehrlich ascites carcinoma cells (EAC; 1×10^6 cells/animal, ip) and were kept as tumour bearing control. Group III animals were fed with AC II (1g/kg body weight, orally) for 5 consecutive days. Group IV animals received AC II (1g/kg body weight, orally) for 5 consecutive days and EAC (1×10^6 cells/animal) was given intraperitoneally after the last dose of drug. 24h after tumour induction the animals were sacrificed at different time points. Spleen cells were processed into single cell suspension and used as effector cells for NK and ADCC activity.

A short-term 4hr chromium (^{51}Cr) release assay⁷ was performed in RIA tubes for determining the Natural Killer cell activity. Chromium labeled K562 cells (target cells) were incubated for 4hr at 37°C with effector cells to obtain the effector-target ratio 100:1. The released radioactive Chromium in the supernatant was counted using a gamma ray spectrometer. The experiment was set in triplicates and the percentage of cell lysis was calculated as:

Percentage specific cell lysis =

$$\frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Total release} - \text{Spontaneous release}} \times 100$$

A short-term 4hr chromium (^{51}Cr) release assay⁷ was performed in RIA tubes for determining the ADCC activity. Chromium labeled SRBC was used as the target cells in ADCC assay and spleen cells from animals of various groups as the effector cells. Anti-SRBC antibody was raised in rabbits and was used as the source of antibody in the ADCC assay. Percentage of cell lysis was calculated as described above.

Blood was collected from the above animals by heart puncture and sera was separated. It was inactivated at 56°C for 30 min and used as anti-EAC antibody for the determination of ACC by trypan blue exclusion method⁸.

Determination of CTL activity — The stimulatory effect of AC II on cytotoxic T lymphocytes was determined in BALB/c mice by the Winn's Neutralization assay using EL4 thymoma cells as target cells⁹. Spleen cells (2×10^7 cells) from C57BL/6 were injected to BALB/c mice subcutaneously. These animals were further treated with or without AC II (1g/kg body weight, 5 doses, orally). Spleen cells (1×10^7 cells) from these mice which act as effector cells were mixed with EL4 cells (5×10^5 cells) which are CTL sensitive cells, and incubated for 1hr at 37°C in 5% CO₂ atmosphere. Further, the EL4 cells were injected intraperitoneally to different groups of animals (6 animals/group) to develop ascites tumour

Group I : EL4 alone

Group II : EL4 cells + spleen cells from untreated animal

Group III : EL4 cells + spleen cells from AC II (1g/kg body weight) treated animal

All the animals were observed for the survival for 70 days.

Statistical analysis — Data were expressed as mean $\pm$ SD. Significance levels were compared by one way ANOVA test followed by Dunnett's test and $P < 0.05$ were considered significant using Graphpad Instat 3 software.

Results

Effect of AC II on lymphocyte proliferation — Administration of AC II significantly enhanced the proliferation of lymphocytes in presence of mitogen as seen by increased thymidine uptake (Table 1). Thymidine uptake by the lymphocyte was significantly enhanced by incubation with PHA or Con-A. Administration of ACII to animals significantly increased thymidine uptake. Addition of PHA and Con A to ACII treated cells further increased the thymidine uptake indicating increased maturation of lymphocytes by treatment with ACII.

Effect of AC II on cytokine levels — AC II administration increased the levels of cytokine IL-2 almost 3 times (Table 2). IL-2 is a cytokine with multi-function and involved in the tumour cell lysis. Stimulation of IL-2 may be responsible for the cell

mediated immune response produced by ACII. There was also significant increase in the TNF- α value, which is pro-inflammatory cytokine and involved in lytic action. Interferon- γ was also found to be increased by ACII administration but the increase was not significant. These results indicate that ACII has significant effect on the cytokines involved in the cell mediated immune response.

Effect of AC II on NK cell activity — The NK cell activity was found to increase progressively from 48 h after drug treatment (Fig. 1). The maximum NK cell activity was observed on 7th day after AC II treatment with a percentage of cell lysis of 41% for AC II treated animals and 42% for AC II treated tumour bearing mice. In untreated tumour bearing mice the maximum NK cell activity was observed on 9th day after tumour inoculation with a percentage cell lysis of 19% and in normal animals it was found to be 2.04%.

Effect of AC II on ADCC activity — ADCC was found to be enhanced in normal and tumour bearing mice after AC II treatment (Fig. 2). Maximum ADCC

Table 1 — Effect of AC II on Lymphocyte proliferation

[Values are mean $\pm$ SD]

Group	H ³ Thymidine uptake (counts per min.)
Normal spleen alone	1687 $\pm$ 1475
Normal spleen + PHA (2.5 μ g/ml)	3702 $\pm$ 194
Normal spleen + Con A (10 μ g/ml)	4400 $\pm$ 150
AC II spleen alone	4615 $\pm$ 196
AC II spleen + PHA (2.5 μ g/ml)	5391 $\pm$ 222
AC II spleen + Con A (10 μ g/ml)	5508 $\pm$ 120

The animals were sacrificed after drug treatment and spleen cells were used for lymphocytes. Spleen cells were incubated with mitogens like PHA or Con A. The amount of radioactive thymidine incorporated to DNA was counted using liquid scintillation counter.

Table 2 — Effect of AC II on cytokine levels in normal mice

[Values are mean $\pm$ SD]

Group	IL-2 (pg/ml)	IFN- γ (pg/ml)	TNF- α (pg/ml)
Normal	7.87 $\pm$ 1.5	2956.77 $\pm$ 92.3	24.01 $\pm$ 2.8
AC II 1 g/kg body weight	25.07 $\pm$ 1.2**	3186 $\pm$ 122.0	30.08 $\pm$ 1.1

** $P < 0.01$

After 5 doses of drug treatment, blood was collected from the animals, serum separated and cytokines such as IL-2, IFN- γ and TNF- α was determined by ELISA method.

activity was observed on 13th day with percent cell lysis of 42% for AC II treated normal mice and 44% for AC II treated tumour bearing animals. In untreated tumour bearing animals the maximum ADCC activity was observed on day 15th after tumour inoculation and the percentage of cell lysis was found to be 15%. In normal animals the maximum percentage of cell lysis was found to be 2.8%.

Effect of AC II on ACC activity — ACC was also enhanced after treatment with AC II (Fig. 3) in normal and tumour bearing mice. Maximum cell lysis was observed on 13th day for AC II treated animals (23%) and AC II treated tumour-bearing mice (24%). While in tumour bearing control animals, the maximum cell lysis (14%) was observed on 17th day. In normal animals ACC was found to be 2% on 9th day.

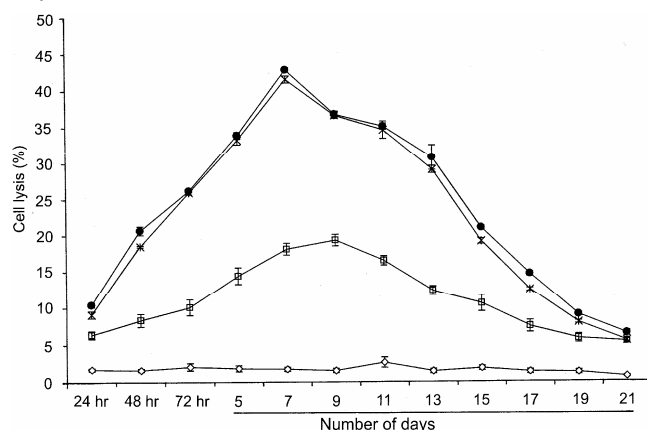


Fig. 1 — Effect of AC II on natural killer cell activity of normal and ascites tumour bearing mice [—◇— normal; —□— tumor alone; —×— AC II 1 g/kg bodyweight; —●— AC II 1 g/kg bodyweight + tumor]

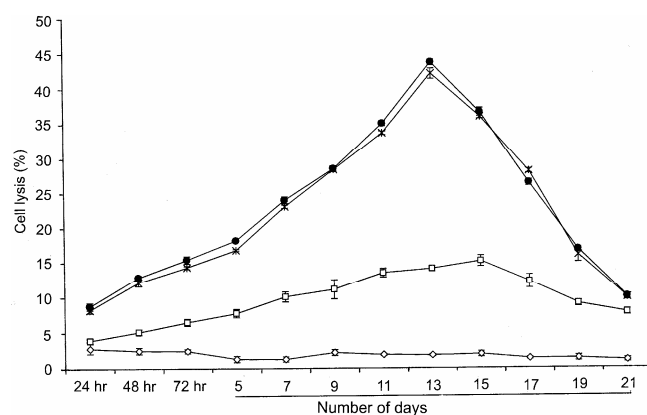


Fig. 2 — Effect of AC II on ADCC of normal and ascites tumour bearing mice [—◇— normal; —□— tumor alone; —×— AC II 1 g/kg bodyweight; —●— AC II 1 g/kg bodyweight + tumor]

Effect of AC II on CTL activity — After EL4 inoculation in control group all mice died within one month and mean survival rate was found to be 26 days (Table 3). In animals injected with EL4 cells + normal spleen cells, the survival rate was found to be 39 days and in EL4 + AC II treated spleen cells mean survival rate was found to be 62 days. Increase in the life span was 138 %.

Discussion

Administration of AC II has been reported to increase production of circulating antibody titre and antibody forming cells in normal mice⁶. Present studies indicate that the treatment with ACII could stimulate the proliferation of immune cells, as seen by a marked enhancement in the proliferation of spleen cells in presence of mitogens such as PHA and ConA.

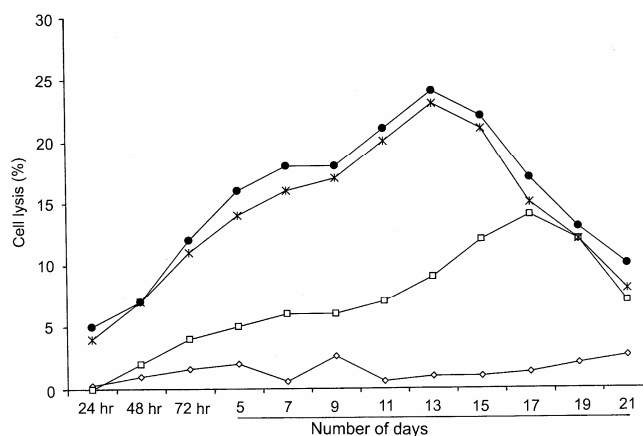


Fig. 3 — Effect of AC II on ACC of normal and ascites tumour bearing animals [—◇— normal; —□— tumor alone; —×— AC II 1 g/kg bodyweight; —●— AC II 1 g/kg bodyweight + tumor]

Table 3 — Effect of AC II on CTL activity

[Values are mean $\pm$ SD. Figures in parenthesis are percentage survival of EL4 thymoma bearing animals.]

Group	CTL activity
EL4 alone	26 $\pm$ 2.19 (—)
EL4 + Normal Spleen	38.8 $\pm$ 1.17 (49.2)
EL4 + AC II 1g/kg body weight	62 $\pm$ 3.03 (138)

The stimulatory effect of AC II on cytotoxic T lymphocytes was determined in BALB/c mice by the Winn's Neutralization assay using EL4 thymoma cells as target cells. Survival rate was observed for 70 days.

Administration of AC II enhanced the activity of non-specific immune cells such as NK cells in normal and tumour bearing mice. Animals with augmented NK cell activity display increased resistance to the development of tumour and metastasis¹⁰. It is believed that tumour development; outgrowth and metastasis are under the surveillance of the immune system. NK cells have also been implicated in tumour surveillance and tumour cell lysis. They are also shown to lyse variety of tumour cell lines *in vitro* as well as *in vivo* using multiple murine cancer models¹¹. Animals with low levels of NK cell activity have been shown to develop an increased number of spontaneous and experimental tumours and their metastases¹². The expression of NK cell activity in normal and tumour bearing mice was found to be enhanced by AC II and was observed much earlier than control animals.

ADCC is the co-operative interaction of humoral and cell mediated immune effectors in the cell-mediated cytotoxicity. In this cytotoxicity model, cellular effectors with receptors for the F_c portion of immunoglobulin molecules produce target cell lysis by attachment to the F_c portion of antibodies bound to target cells via their antigen combining sites. ADCC activity in normal and tumour bearing mice was found to be enhanced by AC II and it was observed much earlier than control animals.

Administration of AC II was found to increase the CTL production in animals. Mean survival rate was significantly increased in AC II treated tumour-bearing animals when compared with EL4 treated group.

The primary activation of NK cells lead to the production of IFN- γ which alone or in combination with IL-2 which is a known T-cell growth factor¹³ stimulate the lytic activity of NK cells¹⁴ which induces antitumour response¹⁵. In this work it has been shown that oral administration of ACII increased the production of IL2, IFN- γ and TNF α indicating the involvement of these cytokines in the activity of ACII.

All these results indicate that AC II can enhance the humoral and cell mediated immune responses in normal mice and its usefulness in HIV could be explained in terms of stimulating immune response to produce target cell lysis. It could also be postulated that ACII may have a significant role in the tumour cell lysis as well, although this has not been experimentally proven.

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