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Immunomodulatory and Antitumor Actions of Medicinal Plant *Tinospora cordifolia* Are Mediated Through Activation of Tumor-Associated Macrophages

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ABSTRACT

The present investigations were under taken to study whether the tumor-associated macrophages (TAM) of Dalton's lymphoma (DL), a spontaneous transplantable T cell lymphoma, can be activated by the alcoholic extract of medicinal plant *Tinospora cordifolia* (ALTC). Intraperitoneal administration of ALTC in DL-bearing mice not only augments the basic function of macrophages such as Phagocytosis as well as their antigen presenting ability and secretion of IL-1, TNF and RNI. The results of the present investigation also indicate that the intraperitoneal administration of ALTC slow down the tumor growth and increases the life span of tumor bearing host, thus showing its anti tumor effect through destabilizing the membrane integrity of DL cells directly or indirectly. This is the first study of it's kind regarding the effect of alcoholic extract of *Tinospora cordifolia* on the activation of tumor associated macrophages and showing the antitumor effect on the spontaneous T-cell lymphoma (DL), thus may have clinical implications.

Key Words: *Tinospora cordifolia*; Tumor-associated macrophages (TAM); RNI; IL-1; TNF; Antigen presentation; Phagocytosis; Arginase activity; Cytotoxicity; Dalton's lymphoma.

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INTRODUCTION

Tumor growth, in general, is invariably associated with the onset of immunosuppression in a tumor-bearing host.[1,2] Along with tumor progression, there is a concomitant suppression of different types of immune responses[2] as well as in the process of hemopoiesis.[3] Recently, biological response modifiers have been attracting much attention because of their anti-tumor effects and their potential to partially or fully restore the tumor-induced immunosuppression.[4]

Macrophages are important effector cells in immune responses to neoplasia.[5,6] Tumor associated macrophages (TAM) play diverse and often-conflicting roles in tumor progression.[7,8] TAM not only contribute to tumor inhibition by exerting cytotoxic and cytostatic response against tumor cells but can also facilitate tumor progression.[9,10] TAM are known to produce a number of factors such as tumor neorosis factor (TNF-α), reactive nitrogen intermediates (RNI), reactive oxygen intermediates (ROI), Arginase, Prostaglandin E, Transforming growth factor β and Interleukin-6 (IL-6),[11–13] which affect tumor growth in either directions.

In the recent years, we have been attempting to elucidate the effect of the progressive growth of Dalton's lymphoma (DL), a transplantable T cell lymphoma of spontaneous origin, on the immune responses of DL-bearing host.[12–19] DL was selected as a model tumor system because murine tumors of spontaneous origin have been reported to resemble with human malignancies most closely.[1] Previous studies from our laboratory have elucidated that the progressive growth of DL resulted in the inhibition of cytotoxic and other accessory functions of TAM.

There has been a considerable interest in identifying and characterizing natural compounds for immunomodulatory and antitumor activity, including agents suct as polysaccharides, phenols and alkaloids.[20,21] The '*rasayana*' of '*Ayurvedic*' medicine constitutes a rich source of active substances for immunotherapy based on herbal preparations. The most popularly used ones are Ocimum *sanctum* (tulsi), *Azadirachta indica* (neem), *Tinospora cordifolia* (guruchi) and *Withania somnifera* (ashwagndha).[22] Tulsi has been reported to possess adaptogenic and antistress activity.[23,24] Neem has been evaluated for their immunostimulating properties.[25,26] *Tinospora cordifolia*, an Indian medicinal plant with powerful immunostimulant activity,[27] has been evaluated as an adjuvant in clinical conditions of some immunodisorders.[22] However, to the best of our knowledge there is no report regarding the immunomodulatory effect of most of the herbal preparations as well as that of *Tinospora cordifolia* on the tumor-induced immunosuppression. In view of these observations, in the present study we investigated if *Tinospora cordifolia* could prolong the survival of DL-bearing mice and if the same could be associated to the immunomodulatory actions of *Tinospora cordifolia*.

MATERIALS AND METHODS

Reagents and Culture Media

Tissue culture medium DMEM and most of the chemicals were purchased from Himedia (Mumbai, India). LPS, Keyhole Limlet Hemocyanin (KLH) and MTT

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[3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Culture medium was supplemented with 20 mg/ml gentamycin, 100 mg/ml streptomycin, 100 IU penicillin and 10% FCS (Himedia) designated as complete medium. All the reagents were free from endotoxin contamination. The cell cultures were carried out at 37°C in a CO_2 incubator (Sheldon, USA) having 5% CO_2 in air in humidified atmosphere.

Mice and Tumor Lines

Inbred, pathogen free BALB/c mice, of either sex at 8–12 week age were used. DL was maintained in ascitic form by serial transplantation in BALB/c mice. The DL cell line is also maintained in vitro culture and in a cryopreserved state for reference purpose. L929 (mouse fibroblast) cell line was obtained from National Tissue Culture facility, Pune, (India). For every experiment mice in a group of six animals each, were transplanted i.p with DL (1×10^5 cells/mouse). TAM were harvested from the mice on the 6th day after the transplantation of DL, designated as early tumor-bearing stage.[13]

Preparation of Alcoholic Extract of *Tinospora cordifolia* (ALTC)

Fresh, shade dried *Tinospora cordifolia* plants were collected and the alcoholic extract was prepared by extraction with 70% ethanol according to the method described by Archana and Namashivayam.[23] 100 ml alcohol was placed in a glass container and 1 g *Tinospora cordifolia* powder was added to it. The suspension was kept in an airtight container at room temperature for 7 days and shaken 5–6 times daily. After 7 days, the supernatant was decanted, filtered and stored. The filtrate was concentrated in a vacuum-evaporator and final product was stored at 4°C until use.

Isolation of Tumor-Associated Macrophages (TAM)

Mice, with DL, were killed by cervical dislocation and peritoneal exudates cells (PEC) were harvested by peritoneal lavage as described earlier.[13] The PEC were cultured in plastic tissue culture flasks (Greiner, Germany) at 37°C in a CO_2 incubator for 2 h. The cultures were then washed thrice with warm serum-free medium with gentle flushing to ensure that all the DL and/or other nonadherent cells were removed. Approximately 95% of the adherent cell population were tumor-associated macrophages as determined by morphology. These TAM were detached from the tissue Culture flask with a cell scraper and plated in a 96 well flat bottom culture plate (1.5×10^5 cells/well).

MTT Assay

MTT assay was carried out to estimate tumor cytotoxicity, antigen presenting ability and IL-1 secretion, following a method described by Mosmann.[28] MTT [3-(4, 5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] was dissolved in PBS at a concentration of 5.0 mg/ml. 50 µl of the MTT solution was added to each well of the culture plate containing 200 µl medium and incubated at 37°C for 4 h. The plate was centrifuged for 5 min at 100 g at 4°C (Remi, India). Supernatant was then removed

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carefully without disturbing the dark blue formazan crystals. 50 µl of DMSO was added to each well and mixed thoroughly to dissolve the crystals of formazan. The plates were then read on a microplate reader (Labsystem, Finland) at a wavelength of 540 nm. Readings were presented as OD at 540 nm.

Assay of TNF Activity

The activity of TNF in the culture supernatant of TAM was measured by dye uptake assay as described earlier.[14] Briefly, 3×10^4 L929 cells, in 100 µl medium were grown in wells of a 96 well tissue culture plate in the presence of 1 µg/ml of actinomycin D and 100 µl of the test culture supernatant. After 18 h of incubation the plates were washed and cell lysis was determined by staining the plate with a 0.5% (w/v) solution of crystal violet in methanol/water (1:4 v/v). The OD was measured at 540 nm. % Cytotoxicity was calculated as follows:

$$\% \text{ Cytotoxicity } = \text{C} - \text{T}/\text{C} \times 100$$

where C is the absorbance of wells containing L929 cells incubated in medium alone, and T is that of those wells in which L929 cells were incubated with culture supernatant of TAM.

Assay for Nitrite Production

Nitrite production in the culture supernatant was determined by a spectrophotometric assay method of Ding et al.,[11] as described earlier.[13] Briefly, 100 µl of sample was collected from the culture supernatants and incubated with an equal volume of Griess reagent (one part of 1% sulfanilamide in 2.5% phosphoric acid plus and part of 0.1% naphthylethylenediamine dihydrochloride in distilled water, were mixed together and used within 12 h of use and kept chilled) at room temperature for 10 min. The absorbance at 550 nm was determined with an automatic ELISA plate reader (Labsystem, Finland). Nitrite concentration was determined by using sodium nitrite as standard. Data were expressed as nitrite release µmol nitrite/1.5×10^5 cells originally plated. In all the experiments, nitrite contents in wells containing medium without cells was also measured and subtracted.

Detection of Inducible NO Synthase (iNOS) Expression by Semi-quantitative Reverse Transcription—Polymerase Chain Reaction (PCR)

Total cellular RNA was isolated from tumor associated macrophages by using the Qiagen RNeasy mini Kit (Batch no. 4061509). The Primer sequences for iNOS used were iNOS forward primer 5′-CACGAATTCGCTTGCCCCTGGA 3′ and for reverses primer 5′-ATAGGATCCTTTGATCCTCACA 3′. Before use, the integrity and purity of RNA was checked by electrophoresis. The RT-PCR reaction was carried out following a method described elsewhere.[29] The reaction mixture contained 1 µl of CDNA, 50 ng of forward and reverse primer sequences of iNOS, 1 × PCR buffer (Genetix), 0.2 mM each of dNTPs (MBI fermentas), 2 mM $MgCl_2$, and 0.5 U of Gold Taq DNA

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Polymerase (Genetix) in a total volume of 20 μl. The PCR was run on a programmable thermocycler (Techne, U.K.) as follows: an initial denaturation step of 5 min at 94°C followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 2 min. The PCR products were resolved on a 1.5-% agarose gel and visualized by ethidium bromide staining and UV irradiation. The gel images were captured on a Gel doc image analysis system (Biorad, Australia).

Assay for Antigen Presenting Ability of TAM

The antigen presenting capacity of the TAM towards Keyhole Limplet Hemocyanin (KLH)-primed T cells was measured according to a method described earlier.[15] Mice were immunized in each footpad with 20 μl of an emulsion of KLH in PBS and Freund's complete adjuvant (100 μg KLH/animal). Ten days after the immunization, a single cell suspension of popliteal lymph nodes was prepared. Cell suspension was allowed to adhere to the plastic surface of tissue culture flask (Greiner, Germany) at 37°C in a CO_2 incubator for 2 h followed by passing of the nonadherent cells over a column of nylon wool to remove the B lymphocytes. TAM were treated with KLH (200 μg/ml) for 6 h at 37°C in a CO_2 incubator followed by treatment with mitomycin-C (1 μg/ml) for 30 minutes. The cultures were then washed and layered with 5×10^5 lymphocytes in 200 μl complete medium and incubated for 72 h in a CO_2 incubator at 37°C. T cell proliferation was measured by the MTT assay. Lymphocyte proliferation is represented in terms of OD values as described above.

Assay of IL-1 Activity

TAM obtained from appropriately treated DL-bearing mice were incubated in vitro as indicated, culture supernatants were harvested and checked for IL-1 activity by a standard thymocyte proliferation assay as described earlier.[12] Thymocytes obtained from 4–8 week-old mice were incubated at a concentration of 1.5×10^6 cells/well in a 96-well plastic tissue culture plate with medium containing suboptimal doses of concanavalin A (1 μg/ml) and 2-mercaptoethanol (2×10^{-5} mol) along with the culture supernatant of TAM. The cultures were then incubated at 37°C in a CO_2 incubator for 72 h. Thymocyte proliferation was measured by MTT assay as described above.

Assay for Arginase Activity

Arginase activity was assayed by a method as described earlier.[12] Culture supernatants (0.5 ml) of macrophages, after respective treatments, were added to centrifuge tubes containing 0.5 ml of 0.424 μM arginine (pH 9.5) and 0.4 ml of 0.1 M Tris buffer (pH 8.0). Reaction mixture was incubated at 37°C for 10 min. The reaction was stopped by adding 1.0 ml of 87% (v/v) acetic acid followed by the addition of 1.5 ml of 0.073 M $Ba(OH)_2$ to remove amino acids and interfering materials as barium salts; 0.4 ml of 0.273 M ZnSO4 was added to this mixture and centrifuged at 1000 rpm for 10 min to separate the precipitate and get a clear supernatant. The supernatant was transferred to the clean separately marked tubes and used for the estimation of urea, as described.

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0.5 ml of the above supernatant was placed in a test tube containing 0.5 ml of distilled water, 1 ml of diacetyl monoxime reagent [1% diacetyl monoxime (w/v) in 5% acetic acid (v/v)]. 2 ml of oxidizing agent (10 mg arsenic acid/ml in concentrated HCl) was added to this mixture and incubated for 30 min in boiling water bath. After cooling at room temperature, the O.D was measured at 475 nm. The results are expressed directly as the O.D/3 $\times$ 10^5 cells.

Phagocytosis

Phagocytosis was determined by the method of Oda and Maeda[30] with modification. Treated or untreated TAM cultured on glass coverslips kept in 35 mm petridish (Tarson, India) were challenged with heat killed yeast cells (2 $\times$ 10^8 cells/ml) for 90 min at 37°C in a CO_2 incubator. The non-phagocytosed yeast cells were washed with warm PBS. The coverslips with TAM were then fixed in methanol for 2 min and stained them with Geimsa stain for 1 hour. Excess stain was washed out under tap water. The coverslips were mounted on a slide with DPX and phagocytosis was examined under light microscope (Leitz, Germany). The number of TAM, which phagocytosed yeast cells, were counted.

Evaluation of Antitumor Effect

The ALTC was injected for 7 days intraperitoneally at a dose of 100 mg/kg body weight and 200 mg/kg body weight in mice after 2 days of tumor transplantation. ALTC suspensions were freshly prepared in PBS, the same volume of PBS was injected in control mice. Six animals were used for each set of experiment. Therapeutic effectiveness of ALTC against tumor-bearing mice was assessed from the T/C percentage, which was calculated as follows:[31]

$$\% \text{ of } T/C = \text{Mean life span of treated mice} / \text{Mean life span of untreated mice} \times 100$$

The antitumor response was also measured as median survival time (days), in which the median life span was determined with the dying animals only. Percentage of increased life span (ILS)[32] was calculated as:

$$\% \text{ of ILS} = (\text{Median survival time of treated group} / \text{Median survival time of control group} - 1) \times 100$$

Osmotic Fragility Test

Osmotic fragility test was performed as described by Roesanu et al.,[33] with some modifications. 2.5 $\times$ 10^6 DL cells were incubated with different concentrations of ALTC or medium alone for 16 h. After incubation the cells were washed with PBS by centrifugation (800 $\times$ g, 10 min). The pellet was resuspended in 1 ml of isotonic PBS or hypotonic PBS at various concentration of NaCl and incubated further at 37°C for

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20 min. Then the PBS was replaced with complete medium and incubated further at 37°C for 10 min. The viability of the cells was checked by MTT assay.

Statistical Analysis

The statistical significance of the difference between the test groups was analyzed by Student's *t*-test (two tailed). All the experiments were done in triplicate and repeated at least three times. In results the values shown are mean ± SD from a representative experiment done in triplicate. In other experiments similar results were obtained.

RESULTS

Effect of In Vivo Administration of ALTC on the IL-1 Production by TAM

The effect of in vivo administration of alcoholic extract of *T. cordifolia* (ALTC) on IL-1 production of TAM was investigated. 0.5 ml of ALTC in PBS at a concentration of 100 or 200 mg/kg body weight or PBS alone was injected intraperitoneally to DL-bearing mice on 3rd, 4th, and 5th day after DL transplantation. TAM were obtained on day 6th after DL transplantation and incubated in vitro for 24 h in the presence or absence of LPS (10 μg/ml) and the culture supernatant was checked for IL-1 activity. Results are shown in Table 1. Little IL-1 activity was observed in culture supernatant of TAM obtained from PBS administered mice on incubation in vitro in medium without LPS. LPS treatment of these TAM enhanced the production of IL-1. TAM obtained from mice administered with PBS containing ALTC 100 mg/kg body weight showed significantly enhanced production of IL-1 in vitro on incubation in medium without LPS as compared to TAM of PBS administered mice treated with LPS in vitro.

Table 1. Effect of in vivo administration of ALTC on IL-1 production by TAM.

	Thymocytes proliferation (OD at 540 nm ± SD)		
	In vivo treatments		
In vitro treatment	PBS	ALTC (100 mg/kg body weight)	ALTC (200 mg/kg body weight)
Medium	0.764 ± 0.068	2.103 ± 0.20**	2.562 ± 0.25
LPS	1.317 ± 0.13*	2.548 ± 0.24*	3.252 ± 0.30**

TAM obtained from DL-bearing mice administered with ALTC or PBS were incubated in vitro in medium alone or containing LPS (10 μg/ml) for 24 h. Cell-free culture supernatants were harvested and assayed for soluble IL-1 activity.
*$p < 0.05$ vs. values for corresponding control of TAM incubated in vitro in medium without LPS.
**$P < 0.05$ vs. values for TAM obtained with ALTC with or without LPS.

IL-1 activity of TAM was further enhanced when mice were administered with high dose of ALTC (200 mg/kg body weight).

Effect of In Vivo Administration of ALTC on the Antigen Presenting Ability of TAM

TAM were obtained from PBS or ALTC administered mice and the antigen presenting ability was studied after treatment in vitro with LPS (10 μg/ml). Results are shown in Figure 1. TAM obtained from tumor-bearing mice administered with PBS showed enhanced antigen presenting ability, on the other hand TAM obtained with ALTC administered mice showed significantly higher antigen presenting ability as compared to TAM obtained from mice administered with PBS alone on incubation in vitro in medium alone or containing LPS.

Effect of In Vivo Administration of ALTC on the Arginase Activity of TAM

Next we investigated the effect of in vivo administration of ALTC on the arginase activity of TAM. TAM obtained from PBS or ALTC administered mice were incubated for 24 h in the presence or absence of LPS (10 μg/ml) and the supernatant were assayed for arginase activity. Results are shown in Figure 2. TAM obtained from mice administered with PBS showed minimal Arginase activity, which could be only marginally altered upon LPS treatment. Similarly, TAM of ALTC administered mice showed a comparable level of arginase activity which could not be significantly altered upon LPS treatment in case of doses of 200 and 300 mg/kg body weight of ALTC, whereas only at a dose of 100 mg/kg body weight the minimal arginase activity of TAM could be significantly augmented upon LPS treatment in vitro.

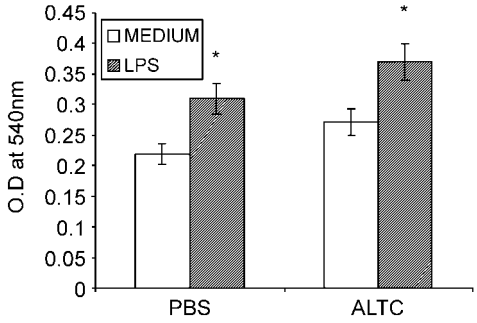

Figure 1. Effect of in vivo administration of ALTC on the antigen presenting ability of tumor associated macrophages. TAM obtained from mice administered with PBS or ALTC (200 mg/kg body weight) were incubated in vitro for 24 h in medium alone or containing LPS (10 μg/ml). Antigen presenting ability was assayed as described in materials and methods. Values are mean ± SD from a representative experiment done in triplicate. In other experiments similar results were obtained. *$p < 0.05$ vs. values for corresponding control.

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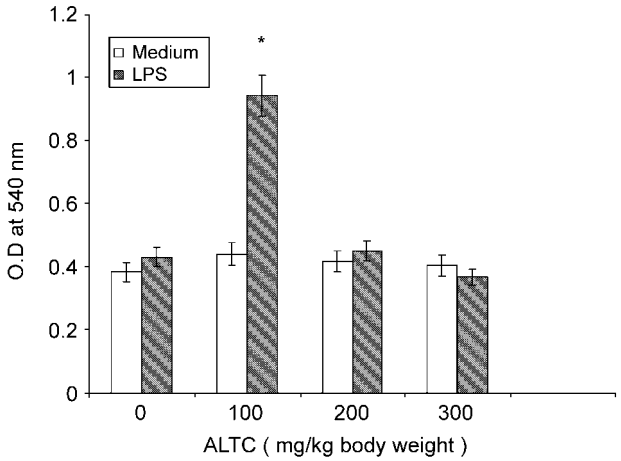

Figure 2. Effect of in vivo administration of ALTC on the arginase activity of TAM. TAM obtained from mice administered with PBS or ALTC (100, 200, 3000 mg/kg body weight) were incubated in vitro for 24 h in medium alone or containing LPS (10 μg/ml). Arginase activity was assayed as described in materials and methods. Value are mean ± SD from a representative experiment done in triplicate. In other experiments similar results were obtained. *$p < 0.05$ vs. values for corresponding control.

Effect of In Vivo Administration of ALTC on the Phagocytotic Activity of TAM

TAM were obtained from PBS or ALTC administered mice and the phagocytotic activity was studied after treatment in vitro with or without LPS (10 μg/ml). As shown in Table 2, TAM obtained from tumor-bearing mice administered with ALTC showed a significantly higher phagocytotic activity as compared to TAM obtained from mice administered with PBS alone on incubation in vitro in medium with or without LPS.

Table 2. Effect of in vivo administration of ALTC on the phagocytic activity of TAM.

	No. of TAM showing phagocytic activity ± SD		
	In vivo treatment		
In vitro treatment	PBS	ALTC (100 mg/kg body weight)	ALTC (200 mg/kg body weight)
Medium	25 ± 2.4	35 ± 3.5	53 ± 5.2
LPS	36 ± 3.5*	53 ± 5.0*	65 ± 6.2*

TAM obtained from DL-bearing mice administered with ALTC or PBS were incubated in vitro in medium alone or containing LPS (10 μg/ml) for 24 h. Yeast cell phagocytosed by the treated or untreated TAM were determined as indicated in the materials and methods.
*$p < 0.05$ vs. values for corresponding control of TAM incubated in vitro in medium without LPS.

Treatment of the TAM in vitro with LPS resulted in a further increase of the phagocytotic activity.

Effect of In Vivo Administration of ALTC on the Production of RNI

The effect of in vivo administration of the alcoholic extract of *T. cordifolia* (ALTC) on RNI production by TAM was studied. ALTC in PBS (200 mg/kg body weight) or PBS alone was injected to normal or tumor-bearing mice on 3rd, 4th and 5th day after DL transplantation. TAM were incubated in medium alone or containing LPS (10 μg/ml) for 24 h and assayed for the production of RNI. Results are shown in Figure 3. TAM obtained from tumor bearing mice administered with PBS alone produced higher amount of NO, and this was further augmented on treatment with LPS. TAM of DL-bearing mice, administered with ALTC, showed an enhanced production of NO, on incubation in vitro in medium alone, as compared to TAM obtained from untreated mice.

Effect of In Vivo Administration of ALTC on the mRNA Expression of iNOS in TAM

As shown above, ALTC induced NO secretion by TAM. In order to determine whether ALTC regulates NO secretion at mRNA level, a reverse transcription Polymerase chain reaction (RT-PCR) was carried out TAM isolated from PBS treated mice did not show expression of iNOS gene. However, TAM isolated from ALTC treated mice showed expression of iNOS gene (Figure 4).

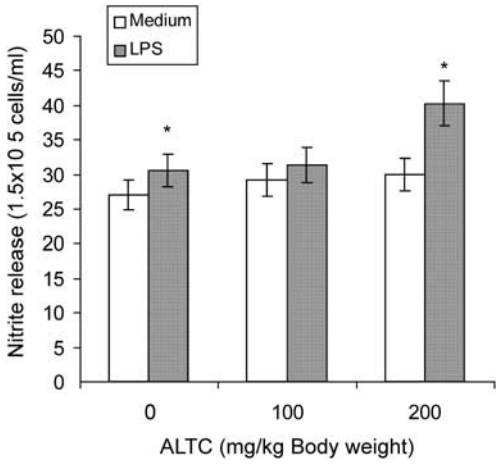

Figure 3. Effect of in vivo administration of ALTC on the production of RNI by TAM. TAM obtained from mice administered with PBS or ALTC (200 mg/kg body weight) were incubated in vitro for 24 h in medium alone or containing LPS (10 μg/ml). No production in culture supernatant was assayed as described in materials and methods. Values are mean ± SD from a representative experiment done in triplicate. In other experiments similar results were obtained. *$p < 0.05$ vs. values for corresponding control.

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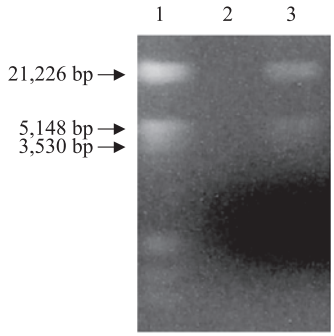

Lane 1: DNA bp ladder.

Lane 2: Control

Lane 3: ALTC treated

Figure 4. Effect of ALTC on iNOS mRNA gene expression. TAM obtained from mice administered with PBS or ALTC (200 mg/kg body weight) were incubated in vitro for 24 h in medium alone or containing LPS (10 μg/ml). mRNA was extracted as described in materials and methods. Data presented for reverse transcription PCR are representative of two experiments. (*View this art in color at www.dekker.com.*)

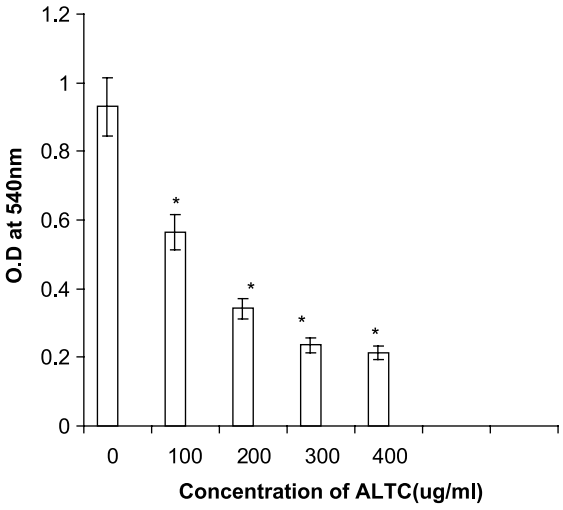

Figure 5. Effect of in vitro treatment of ALTC on the proliferative ability of DL cells. DL cells were incubated for 72 h with different concentration of ALTC (ug/ml) and the proliferative ability was assayed as indicated in materials and methods. Values are mean ± SD from a representative experiment done in triplicate. In other experiments similar results were obtained. *$p < 0.05$ vs. values for corresponding control.

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Table 3. Effect of ATLC on the production of TNF by TAM.

	Cytotoxicity against actinomycin-D treatment L929 cells (%)		
	In vivo treatments		
In vitro treatments	PBS	ALTC (100 mg/kg B.wt)	ALTC (200 mg/kg B.wt)
Medium	5 ± 0.4	10 ± 0.9*	11 ± 1.0*
LPS	10 ± 0.8	21 ± 1.9	36 ± 3.5

TAM obtained from DL-bearing mice administered with ALTC or PBS were incubated in vitro in medium alone or containing LPS (10 μg/ml) for 24 h. Culture supernatants were harvested and assayed for cytotoxicity against actinomycin-D treated L929 cells as described in materials and methods. The values are ± S.D. of a representative experiment done in triplicate. Similar results were obtained in three independent experiments.
*$p < 0.05$ vs. values for corresponding control of TAM incubated in vitro in medium without LPS.

Effect of In Vitro Treatment of ALTC on the Proliferative Ability of DL Cells

DL cells (1×10^5 cells/well) were incubated without or with the indicated doses of ALTC for 72 hr and proliferation of DL cells was estimated by MTT assay. Results are shown in Figure 5. A dose dependent decrease in the proliferative ability of DL cells was observed upon treatment with ALTC.

Effect of In Vivo Administration of ALTC on the Production of TNF by TAM

TAM obtained from PBS or ALTC administered mice were incubated for 24 h in the presence or absence of LPS (10 μg/ml) and the supernatant were assayed for

Table 4. Effect of in vitro treatment of ALTC on osmotic fragility of DL cells.

	% Cytotoxicity			
	Concentration of NaCl (M)			
Treatment	0.12	0.10	0.06	0.02
0 μg/ml	15.4	16.2	23.3	20.0
100 μg/ml	30.0	20.58	14.53	25.30
200 μg/ml	27.6	31.2	21.2	14.2
400 μ/ml	39.0	25.0	17.0	19.0

DL cells were cultured with the indicated concentrations of ALTC in vitro for 16 h and the osmotic fragility was conducted as indicated in the materials and methods. The values are mean of a representative experiment done in triplicate.

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production of TNF, results as shown in Table 3, TAM isolated from DL-bearing mice administered with ALTC showed significant TNF. Production, even in the absence of LPS treatment in vitro. Treatment of these TAM in vitro with LPS resulted in further augmentation of TNF production.

Osmotic Fragility Test

The effect of ALTC on the osmotic property of the DL cells was checked. The results are shown in Table 4. The DL cells were cultured with the indicated concentrations of ALTC in vitro for 16 h and the osmotic fragility assay was conducted. It was found that the DL-cells incubated with ALTC show higher osmotic fragility, while the DL cells incubated in medium alone showed a comparatively lower osmotic fragility. The in vitro treatment of ALTC at the dose of 400 μg/ml showed maximum augmentation of DL cell osmotic fragility.

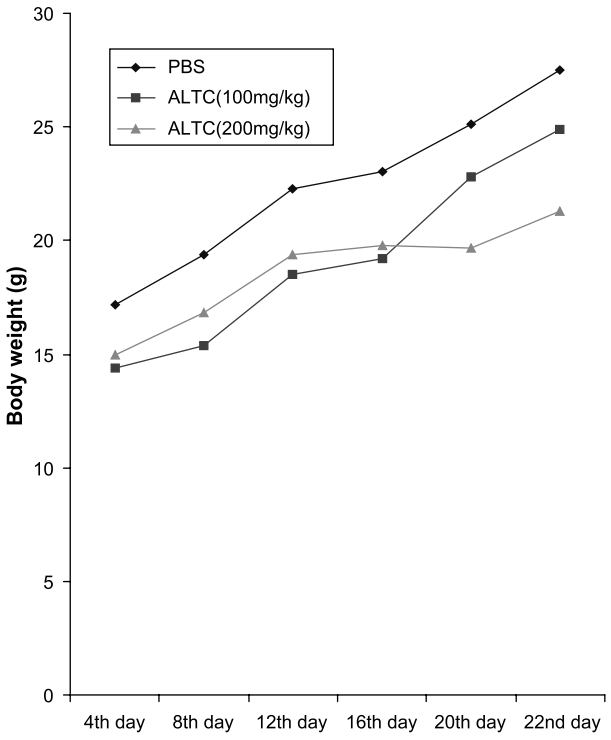

Figure 6. Effect of in vivo administration of ALTC on growth kinetics of DL-bearing mice. Body weight of DL bearing mice after administration of ALTC (100, 200 mg/kg body weight) or PBS was taken on different interval of time as indicated in materials and methods. Values are mean ± SD from a representative experiment done in triplicate. In other experiments similar results were obtained. (*View this art in color at www.dekker.com.*)

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Table 5. Antitumor activity against Dalton's lymphoma.

Treatment	Mean life span of nonsurvivors T/C (days)	% (T/C)	ILS (%)
ALTC (100 mg/kg body weight)	42/30	140	40
ALTC (200 mg/kg body weight)	48/30	160	60

T = tumor bearing; C = Control; ILS = Increased life span.

Effect of In Vivo Administration of ALTC on the Growth Kinetics of DL-Bearing Mice

The effect of in vivo administration of ALTC on the growth kinetics of DL was investigated. DL-bearing mice were administered PBS alone or containing the indicated dose of ALTC after two days of DL transplantation. Weight of DL-bearing mice indicating the growth of tumor was taken on the indicated days. Results are shown in Figure 6. The increase in weight of DL-bearing mice was higher in case of PBS injected group whereas in case of ALTC administered mice the increase in weight was lower.

%T/C an indication of the therapeutic effectiveness of an antitumor compounds and ILS was also calculated. Results are shown in Table 5. It has been reported that a T/C value of 115 indicates significant activity.[31] ALTC at both the doses showed %T/C higher than 115. The values of increased life span also showed similar effects.

DISCUSSION

Although *Tinospora cordifolia* has been shown to augment some of the macrophage functions.[23,34,35] Its effect on the function of TAM remains unclear. It also remains to be determined if *Tinospora cordifolia* has any direct or indirect influence on tumor growth. The results of the present study address some of these problems. It was observed that in vivo administration of *Tinospora cordifolia* resulted in an augmentation of the phagocytic ability of TAM. Phagocytosis is one of the basic and fundamental function of macrophage to clear dead cell debris and non self components.[36] Phagocytosis is also the one of the primary step in the process of antigen uptake by macrophage leading to antigen presentation.[36] Indeed, we observed that the antigen presenting ability of TAM obtained from mice administered with *Tinospora cordifolia* was enhanced. We have previously reported that progressive growth of DL results in inhibition of antitumor functions and endotoxin responsiveness of TAM.[37] It was further shown that TAM were inhibited by mechanisms involving suppressor molecule(s) secreted/induced by tumor cells. Although the mechanisms of the reversal of tumor growth associated effects on TAM upon administration of *Tinospora cordifolia* are not clear but some of the possibilities can be considered. One of the actions of *Tinospora cordifolia* could be direct on macrophage activation.

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Indeed, we show that *Tinospora cordifolia* not only enhanced LPS responsiveness of TAM but could also activate TAM even in the absence of LPS stimulation in vitro. This suggests that *Tinospora cordifolia* could alone suffice all the signals required for activation of TAM. These activated TAM not only showed an enhanced phagocytosis and antigen presenting ability but could also produced enhanced amount of IL-1, which is a co-stimulatory signal for lymphocyte proliferation indeed due to antigen presentation by macrophages. It has been demonstrated that tumor rejection or growth is associated with fate of arginase metabolism through NO synthase or arginase pathways in TAM.[38] Although we showed an up-regulation of IL-1 production but a simultaneous increase in arginase activity was not observed upon in vivo administration of *Tinospora cordifolia*. The observation of the present study also shows that *Tinospora cordifolia* administration results in enhanced RNI production by TAM. On the basis of the findings of the RT-PCR of the present study it is suggested that ALTC regulates NO accumulation in TAM, and it upregulates the expression of iNOS mRNA. Therefore we believed that the increased NO production by ALTC is regulated at the level of transcription of iNOS gene. Moreover, we had previously reported that TAM during progression of DL gets gradually switched from NO synthase pathway to arginase pathway of arginine metabolism, resulting in promotion of tumor growth.[12] However, the observation showing upregulation of IL-1 and RNI but not of arginase activity upon *Tinospora cordifolia* administration indicate that T. cordifolia interferes with tumor growth associated ''switching on'' of the arginase pathway. These conclusion are further supported by our observation that in vivo administration of *Tinospora cordifolia* resulted in prolongation of survival of DL-bearing mice along with a slowing down of the growth kinetics of tumor. It could also be possible that *Tinospora cordifolia* could act not only on TAM but also on the DL cells directly. Indeed the results of the experiment where DL cells were incubated in vitro with ALTC support this suggestion because this extract could inhibit DL cell proliferation and induce DL cell killing by enhancing the osmotic fragility of tumor cells. These results thus indicate that one of the modes of antitumor actions of ALTC on DL cells, was through the destabilization of the tumor cells membrane integrity. This conclusion is also supported by the study of Thatte et al. who showed that some medicinal plants can induce apoptosis in tumor cells.[39] Break down of membrane integrity is one of the hall mark features of apoptosis.[40] This Study demonstrate for the first time that *Tinospora cordifolia* has a dual actions in tumor-bearing mice where on one hand it can activate the complete antitumor potential including the upregulation of basic functions like phagocytosis and antigen presenting ability to secretion of antitumor factors RNI, IL-1, and TNF, while on the other hand it can also directly induce tumor cell cytotoxicity and cytostasis. This study will thus go long way in designing immunotherapeutic protocol for treatment of cancer using the medicinal plant *Tinospora cordifolia*.

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