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Immunomodulatory effect of *Tinospora cordifolia* in tumor-bearing host

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SUMMARY

The present investigation was undertaken to study whether tumor-associated macrophages of Daltons lymphoma (DL), a spontaneous transplantable T cell lymphoma can be activated to tumoricidal state by alcoholic extract of *Tinospora cordifolia* (ALTC). In vivo administration of ALTC (200 mg/kg body weight) in DL-bearing mice resulted in an enhanced RNI production and an augmented cytotoxic response of tumor-associated macrophages. Earlier we had reported that DL-bearing mice show a regression of thymus and an enlargement of spleen. In vivo administration of ALTC to DL-bearing hosts resulted in a decrease in the weight of spleen and counts of splenocytes along with an increase in the weight of thymus as compared to control DL-bearing mice. In vivo administration of ALTC in DL-bearing mice also resulted in an increase in the proliferation of splenocytes/thymocytes and BMC. The results of this study indicate that the ALTC upon in vivo administration in DL-bearing shows immuno-modulatory effects and thus may have clinical significance.

Key words: *Tinospora cordifolia*; Tumor-associated macrophages; Nitric oxide; Dalton's lymphoma; Cytotoxicity

INTRODUCTION

Tumor growth, in general, is invariably associated with the onset of immunosuppression in a tumor-bearing host (Singh *et al.*, 1997; Ben-Effraim S, 1999). Along with tumor progression, there is a concomitant suppression of different types of immune responses (Singh *et al.*, 1997) as well as in the process of hemopoiesis (Parajuli *et al.*, 1995). 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 (Oldham *et al.*, 1983). There has been a considerable interest in identifying and characterizing natural compounds for immunomodulatory activity. They include compounds such as polysaccharides, phenols and alkaloids (Engles *et al.*, 1992; Ingolfsdottir *et al.*, 1994). 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* (ashwagandha). Tulsi has been reported to possess adaptogenic and antistress activity (Prashar *et al.*, 1995; Archana *et al.*, 2000). Neem has been evaluated for their immunostimulating properties (Upadhyay *et al.*, 1992; SaiRam *et al.*, 1997). *Tinospora cordifolia* an Indian medicinal plant with powerful immunostimulant activity (Thatte *et al.*, 1989) has been evaluated as an adjuvant in clinical conditions of some immuno-disorders (Dahanukar *et al.*, 1988). However, to the best of our knowledge, there is no report regarding the immuno-modulatory effect of herbal preparations in general and that of *Tinospora cordifolia* in particular on the tumor-induced immunosuppression.

Macrophages are important effector cells in host immune responses to neoplasia (Adams *et al.*, 1984; Foss *et al.*, 2002). Tumor associated macrophages (TAM) play a diverse and often- conflicting roles in tumor progression (Bingle *et al.*, 2002; Chu *et al.*, 2002). TAM not only contributes to tumor inhibition by exerting cytotoxic and cytostatic response against

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tumor cells but can also facilitate tumor progression (Mantovani *et al.*, 1992; Mantovani *et al.*, 1993). In the recent years, we have been attempted to elucidate the effect of progressive growth of Dalton's lymphoma (DL), a transplantable T cell lymphoma of spontaneous origin, on the immune responses of DL-bearing host (Kumar *et al.*, 1994; Kumar *et al.*, 1995; Parajuli *et al.*, 1995; Kumar *et al.*, 1996; Parajuli *et al.*, 1996; Parajuli *et al.*, 1997; Shanker *et al.*, 2000). DL was selected as a model tumor system because murine tumors of spontaneous origin have been reported to resemble with human malignancies most closely (Ben-Effraim S, 1999). 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 and rendered them unresponsive to biological response modifiers (Parajuli *et al.*, 1996; Parajuli *et al.*, 1997). Despite the reports concerning the immuno-modulatory actions of *Tinospora cordifolia*, it remains to be determined if it can also activate normal and tumor-associated macrophages for production of reactive nitrogen intermediates (RNI) and tumoricidal activity. In view of the lacuna mentioned the present investigation was undertaken to study the effect of *Tinospora cordifolia* on the activation of normal macrophages and tumor-associated macrophages (TAM) isolated from early tumor-bearing stage.

MATERIALS AND METHODS

Reagents and culture media

Tissue culture medium DMEM and most of the chemicals were purchased from Himedia (Mumbai, India). Lipopolysaccharide (LPS) was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All culture media were supplemented with 20 mg/ml gentamycin, 100 mg/ml streptomycin, 100 IU penicillin and 10% FCS (Himedia). 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. Dalton's lymphoma (DL), a spontaneous tumor of thymus 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. For each experiment mice in a group of six animals each, were transplanted i.p with DL (1×10^5 cells/mouse). TAM was harvested from the mice on the 6^{th} day after the transplantation of DL, designated as early tumor-bearing stage (Parajuli *et al.*, 1997).

Preparation of alcoholic extract of tinospora cordifolia (ALTC)

Fresh, shade-dried whole *Tinospora cordifolia* plants were collected and the alcoholic extract was prepared by extraction with 70% ethanol at room temperature (Archana *et al.*, 2000). 100ml alcohol was placed in a glass container and 1g *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) and normal macrophages (NMO)

Mice, with or without DL, were killed by cervical dislocation and peritoneal exudate cells (PEC) were harvested by peritoneal lavage as described earlier (Parajuli *et al.*, 1997). The PEC was cultured in plastic tissue culture flasks (Greiner, Germany) at 37°C in a CO_2 incubator for 2h. 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 was macrophages as determined by morphology. These TAM or NMO 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 *et al.*, 1983). 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. Medium was then removed 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 570 nm. Readings were presented as OD at 570 nm.

Assay for macrophage-mediated tumor cytotoxicity

Macrophage-mediated tumor cytotoxicity was assayed by measuring the killing of target DL cells as described earlier (Parajuli *et al.*, 1997) with some modifications. DL (1.5×10^4 cells/well) were coincubated with TAM at an E: T cell ratio of 10:1. After 24 h the incubation was terminated and MTT assay was carried out and percent cytotoxicity was calculated by the following formula-

$$\% \text{ Cytotoxicity} = \frac{\text{OD of DL cells Cultured with TAM}}{\text{OD of DL Cells cultured alone.}} \times 100$$

Assay for nitrite production

Nitrite production in the culture supernatant was determined by a spectrophotometric assay method of (Ding *et al.*, 1988). Briefly, 100ml 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 mmol 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.

Proliferation assay

Cell proliferation was assayed according to method described earlier with slight modification (Parajuli *et al.*, 1995). BMC, thymocytes or splenocytes obtained by methods described earlier (Parajuli *et al.*, 1995; Kumar *et al.*, 1996; Parajuli *et al.*, 1997; Shanker *et al.*, 2000) were seeded (1×10^6 cells/well), in 100 µl complete medium, in a 96 well tissue culture plate with or without the mitogen concanavalin A (ConA) (2 µg/ml) and incubated at 37°C in a CO_2 incubator for 72h. The proliferation was measured by MTT assay.

Statistical analysis

The statistical significance of the differences between the test groups was analyzed by a student's *t* test (two tailed). All the experiments were done in triplicate and repeated at least three times.

RESULTS

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 macrophages 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 or NMO 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 Fig. 1. TAM obtained from tumor bearing mice administered with PBS alone produced higher amount of NO than NMO, and this was further augmented on treatment with LPS. TAM of DL-bearing mice and NMO of normal mice, administered with ALTC, showed an enhanced production of NO, on incubation *in vitro* in medium alone, as compared to NMO or TAM obtained from untreated mice. The RNI production of NMO of ALTC-administered mice was significantly augmented upon in vitro incubation with LPS.

Effect of *in vivo* administration of ALTC on TAM-mediated tumor cytotoxicity

The effect of in vivo administration of ALTC on TAM- or NMO-mediated cytotoxicity was investigated following the same protocol of the drug administration as described in section 3.1. TAM or NMO, obtained from mice administered with PBS or ALTC, were incubated in vitro in medium alone or containing LPS (10 µg/ml) for 24 h followed by co incubation

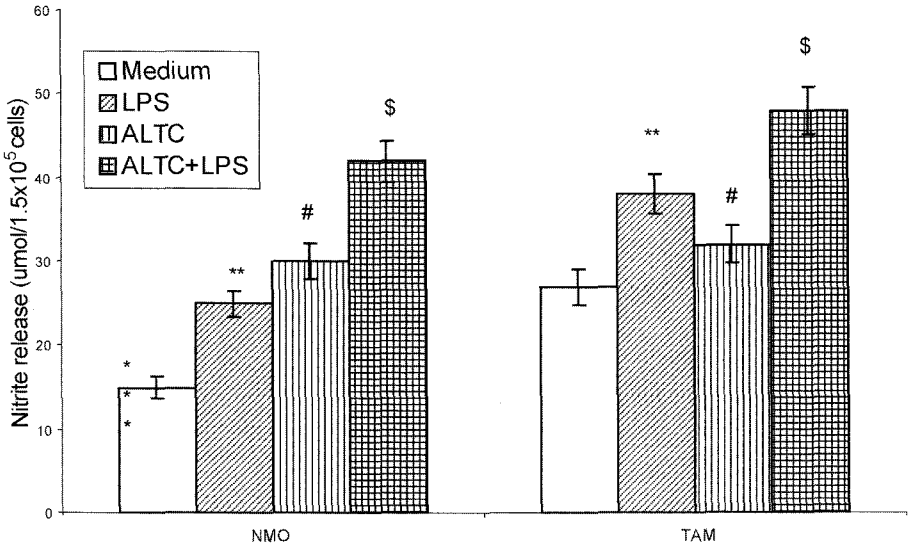

Fig. 1. TAM or NMO obtained from DL-bearing or normal mice administered with PBS or ALTC (200 mg/kg body weight) were incubated with or without LPS (10 μg/ml) for 24 h. Culture supernatants were harvested and assayed for nitrite production. Values are mean ± SD from a representative experiment done in triplicate. In other experiments, similar results were obtained. **$p<0.05$ vs. values for macrophages incubated in medium alone. #$p<0.05$ vs. values for macrophages obtained from mice not administered with ALTC and incubated *in vitro* in medium alone. \$$p<0.05$ vs. values for macrophages obtained from mice administered with PBS or ALTC and incubated *in vitro* in medium alone.

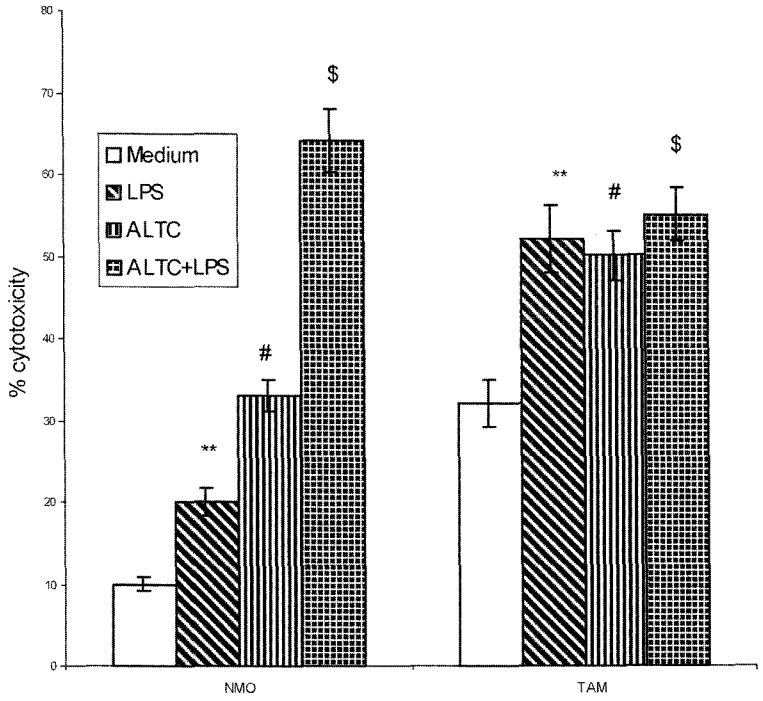

Fig. 2. TAM or NMO obtained from DL-bearing or normal mice administered with PBS or ALTC (200 mg/kg body weight) were incubated *in vitro* with or without LPS (10 μg/ml) for 24h. Percentage cytotoxicity against DL cells was assayed. Values are mean±SD from a representative experiment done in triplicate. In other experiments, similar results were obtained. **$p<0.05$ vs. values for macrophages incubated in medium alone. #$p<0.05$ vs. values for macrophages obtained from mice not administered with ALTC and incubated *in vitro* in medium alone. \$$p<0.05$ vs. values for macrophages obtained from mice administered with PBS or ALTC and incubated *in vitro* in medium alone.

with DL cells (1×10^4 cells/well) for 18 h for the tumor cytotoxicity assay. Results are shown in Fig. 2. TAM obtained from tumor-bearing mice had a significantly higher tumor cytolytic activity than NMO, which further increased upon *in vitro* treatment with LPS. TAM obtained from mice administered with ALTC showed a significant increase in tumor cytotoxicity as compared to NMO. *In vitro* LPS treatment of TAM of ALTC-treated DL-bearing mice did not further augment the tumor cytotoxicity while the same was significantly enhanced in case of NMO of *T.Cordifolia* treated mice.

Effect of in vivo administration of ALTC on the weight of spleen and thymus and on their cell count

We have previously shown that progressive growth of DL results in the regression of thymus (Shanker *et al.*, 2000) and an enlargement of spleen (Kumar *et al.*, 1996), with implications in tumor-induced immunosuppression. Therefore, in the present investigation we were also interested to investigate if DL growth associated aforesaid effects on thymus and spleen could be reversed on *in vivo* administration of ALTC (Table 1). The weight of thymus and count of thymocytes of DL-bearing mice administered with ALTC was found to be significantly higher than that of DL-bearing mice administered with PBS alone. On the other hand, mice administered with ALTC were found to have spleen, which were nearer to normal size with a decreased in the count of splenocytes as compared to that of DL-bearing mice not administered with *T. cordifolia.*

Effect of *in vivo* administration of ALTC on the proliferation of splenocytes, thymocytes and Bone marrow cells (BMC)

Single cell suspension of splenocytes, thymocytes or BMC (1×10^6 cells/well) of DL-bearing mice administered with ALTC or PBS alone were cultured in vitro in the presence of ConA for 72h and proliferation was estimated by MTT assay. Results are shown in Fig. 3. Administration of ALTC to DL-bearing was found to increase the proliferation of splenocytes, thymocytes and BMC *in vitro.* Increase in the proliferation of splenocytes was of 1.65 fold whereas for that of BMC and thymocytes was of 0.9 & 1.43 folds respectively as compared to that of DL-bearing mice administered with PBS alone.

DISCUSSION

We have previously reported a wide range of immunosuppressive actions on the progressive growth of DL (Kumar *et al.*, 1996; Parajuli *et al.*, 1996; Parajuli *et al.*, 1997). The next aim was, therefore, to design and develop an effective immunotherapeutic protocol in DL-bearing hosts to reverse the tumor-induced immunosuppression. There are few reports regarding the immunomodulatory action of *Tinospora cordifolia,* however, it remains unclear if this drug has similar effects in a tumor-bearing host, where the immune system is in a highly suppressed state. Therefore, in the present study we investigated the effect of *in vivo* administration of *T.Cordifolia* on various cell-mediated immune responses of a tumor-bearing host. *In vivo* administration of *T.Cordifolia* was found to activate both NMO and TAM to tumoricidal state, with the magnitude of the macrophage-mediated tumor cytotoxicity being higher than that obtained with NMO of the treated normal mice. TAM has been reported to be highly resistant to activation of signal of LPS & IFN-γ (Parajuli *et al.*, 1997). The mechanism of the

Table 1. Effect of *in vivo* administration of ALTC on the wet weight of spleen, Thymus and counts of splenocytes and thymocytes in DL-bearing mice

Treatments	Wet weight of spleen (mg)	Counts of splenocytes ($\times10^6$cells/ml)	Wet weight of Thymus (mg)	Counts of thymocytes ($\times10^6$cells/ml)
PBS	155±14.2	9.6±0.9	50.0±4.8	4.8±0.4
ALTC	130 ± 13*	6.4±0.64*	62.5±6.1*	9.6±0.9*

The spleen and thymus of PBS or ALTC administered mice were removed, weighed and a single cell suspension of the splenocytes and thymocytes was made. The viable cells were counted using standard trypan blue dye exclusion test [14]. The values mean±SD from a representative experiment.
*$p<0.05$ vs. values of corresponding control

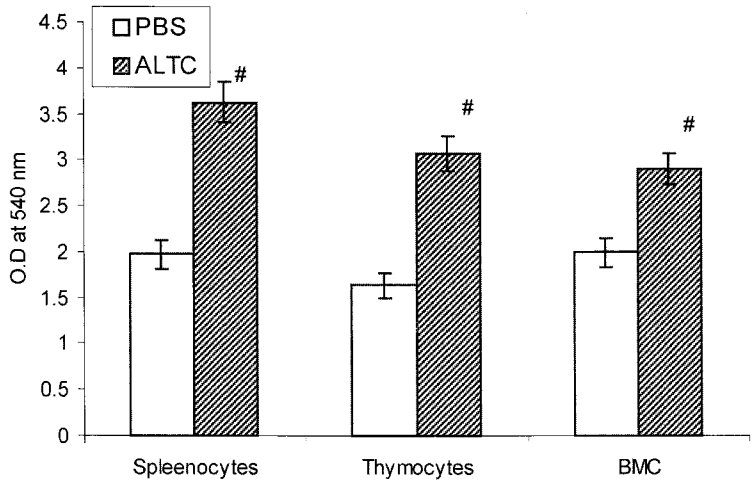

Fig. 3. Splenocytes, thymocytes or BMC of DL-bearing mice were incubated in medium containing ConA (2 μg/ml) for 72 h and proliferation was measured as described in Materials and Methods. Values are mean ±SD from a representative experiment done in triplicate. In other experiments, similar results were obtained. #$p<0.05$ vs. corresponding controls.

activation of TAM by *T.Cordifolia* remains unclear. However, some of the possibilities could be considered. It has been reported that *T.Cordifolia* preparations in crude form or their purified components like cordiosides, cordiofoliosides, and cordiol can directly activate some functions of macrophages (Dahanukar *et al.*, 1988; Dahanukar *et al.*, 2000). Since *T.Cordifolia* was administered in vivo, therefore, the activation of NMO or TAM may not necessarily be only due to a direct effect of *T.Cordifolia* on these cells. The possibility of some indirect mechanism being operative is due to the fact that the proliferative response of splenocytes, BMC and thymocytes is altered in DL-bearing host administered with *T.Cordifolia*. Moreover, DL-associated regression of thymus (Shanker *et al.*, 2000) and spleen enlargement (Kumar *et al.*, 1996) could be reversed by in vivo administration of *T.Cordifolia*. This suggests that *T.Cordifolia* could restore the cytokine homeostasis in DL-bearing host, which is altered along with DL growth (Shanker *et al.*, 2000). Indeed it has been shown that *T.Cordifolia* treatments could enhance production of cytokines (Thatte *et al.*, 1994) like IL-1, which is mitogenic for lymphocytes (Parajuli *et al.*, 1997), and GM-CSF, which has a number of actions on mature and progenitor cells (Parajuli *et al.*, 1995). NO produced in situ by macrophage has also been shown to control lymphocyte blastogenesis (Kumar *et al.*, 1996). An additional possibility is that the observed effects of *T.Cordifolia* could also be the result of tumor regression upon administration of the drug. This was corroborated by the finding that DL-bearing mice on in vivo administration of *T.Cordifolia* show a prolongation of the life span with a significant fraction of the drug-treated tumor-bearing mice showing a complete regression of the tumor (data not shown). It is, therefore suggested that the observed reversal of DL growth could be due to two reasons: firstly, that T. Cordifolia-activated macrophages may cause an enhanced tumor cell death, and secondly, that *T.Cordifolia* could directly kill tumor cells.

Although, further studies will be necessary to work out the precise mode of action of *T.Cordifolia*, prior to the designing a therapeutic protocol using the plant for therapeutic purpose in a tumor-bearing host, the results of the present investigation show for the first time the potential of *T.Cordifolia* to antagonize tumor-associated immunosuppressive actions. These results, therefore, will provide novel direction in the designing effective immuno-therapeutic protocols with *T.Cordifolia*, for treatment of cancer.

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