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Effect of thymus extract on the activation of cytotoxic and accessory functions of tumor-associated macrophages

Pratima Shrivastava, Sukh Mahendra Singh* and Nisha Singh

School of Biotechnology, Banaras Hindu University, Varanasi-221 005, U.P., India

SUMMARY

The present investigation was under taken to study whether the tumor-associated macrophages (TAM) of Daltons lymphoma (DL), a spontaneous transplantable murine T cell lymphoma can be activated to tumoricidal state by crude thymus extract. Intraperitoneal administration of thymus extract to DL-bearing mice resulted in activation of TAM with an enhanced IL-1, TNF and antigen presenting ability. It was found that treatment with thymus extract could also enhance the phagocytic and cytotoxic activity of TAM. However, only a marginal increase in arginase activity was observed. Till date to the best of our knowledge the effect of crude thymus extract on the activation of tumor associated macrophages has not been investigated, this study provides a new piece of information in the area of thymus therapy.

Key words: Tumor-associated macrophages; Daltons lymphoma; IL-1; Antigen presentation; Cytotoxicity; Phagocytosis; Arginase activity

INTRODUCTION

Macrophages play an indispensible role in the host immune responses, to neoplasia (Adams *et al*., 1984; Evans *et al*., 1983; Foss *et al*., 2002). Tumor associated macrophages (TAM) play a diverse and often-conflicting role in the progression of tumor (Bingle *et al*., 2002; Chu *et al*., 2002) TAM not only contribute to tumor inhibition by exerting cytotoxic and cytostatic response against tumor cells but can also facilitate tumor progression (Mantovani *et al*., 1993; Mantovani *et al*., 1992).

In the recent years, we have attempted to elucidate the effect of the progressive growth of Daltons lymphoma (DL), a transplantable T cell lymphoma of spontaneous origin, on the immune responses of DL-bearing mice. DL was selected as a model tumor system because murine tumors of spontaneous origin have been reported to resemble human malignancies most closely (Ben effraim 1999). Previous studies from our laboratory have demonstrated that progressive growth of DL resulted in an inhibition of the cytotoxic and other accessory functions of TAM (Parajuali *et al*., 1996; Parajuali *et al*., 1997). However, it remained unclear if the cytolytic potential of TAM of DL-bearing mice can be partially or fully restored. In order to design immunotherapeutic protocols aimed at activating the TAM for regression of DL, we were interested in screening various naturally occurring biological response modifiers for their potential to activate TAM of the DL-bearing host. We have previously reported that thymus of DL bearing mice involutes along with the progression of DL growth (Shanker *et al*., 2000., 2000: 2000). This may possibly result in a decline in the production of thymic peptides, which could be one of the reasons underlying the inhibited immune responses of the DL-bearing host Up to now more than twenty thymus peptides have been isolated from the entire thymus extract. Thymus extract is known to contain peptides like thymosin a1,thymopoietin, thymulin etc with a wide range of immunomodulatory properties (Bodey *et al*., 2000; Eckert *et al*., 1997; Eckert *et al*., 1997; Garaci., *et al*; 2000). The numbers of clinical examinations that have been made with individual thymic peptides (Kullavanuaya *et al*., 2001; Ohmori

*Correspondence: Sukh Mahendra Singh, School of Biotechnology, Banaras Hindu University, Varanasi-221 005, U.P., India. E-mail: sukh2001@sify.com

et al., 2001; Roy *et al*., 2000) are numerous. In this study we tried to find out whether synergistic action of various thymic peptides that constitute thymic extract, can activate TAM.

MATERIALS AND METHODS

Reagents and culture media

Tissue culture medium DMEM and most of the chemicals were obtained from Himedia (Mumbai, India). Thya1, LPS, MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] and Keyhole Limplet Hemocyanin (KLH) were obtained 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). 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, apparently healthy BALB/c mice, of either sex, of 8-12 weeks of age and 20-25 g body weight were used for experimentation. DL was maintained in ascitic form by serial transplantation in BALB/c mice. The DL cell line was also maintained by serial culture in vitro and in a cryopreserved state for reference purpose. Mice in a group of at least six, were transplanted i.p with DL cells (1×10^5 cells/mouse) for each experimental set. TAM were harvested from the mice 10 days after the transplantation of DL.

Preparation of thymus extract

Thymus extract was prepared following the method described by Loidi *et al*; 1997. Thymic tissue was homogenized under chilled temperature in phosphate buffer saline (9 g/l NaCl; 50 mmol/l phosphate buffer, pH 7.5; EDTA 2 mmol/l) and centrifuged at 10,000×g for 10 min. The supernatant, thus obtained, was centrifuged at 15,000×g for another 5 min. The final supernatant was stored at -20°C until use as thymus extract for various experimental purposes. The protein content in the extract was measured.

Isolation of tumor-associated macrophages (TAM)

TAM were isolated following a method described earlier (Parajuli *et al*., 1997). DL-bearing mice, on the 10th day after DL transplantation or were killed by cervical dislocation and peritoneal exudate cells were harvested by peritoneal lavage using chilled serum free DMEM. Peritoneal exudate cells were 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 other non-adherent cells were fully removed. 95% of the adherent cell population were macrophages as determined by morphology. These TAM were detached from the tissue culture flask gently by scrapping and plated in a 96-well flat bottom culture plates (1.5×10^5 cells/well).

MTT (Tetrazolium) 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 mg/ml. 50 ml 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 formazon crystals. 50 µl of DMSO was added to each well and mixed thoroughly to dissolve the crystals of formazon. 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 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 (Kumar *et al*., 1995). Mice were immunized in each footpad with 20 µl of an emulsion of KLH in PBS and Freunds complete adjuvant (100 µg KLH/animal). Ten days after the immunization, popliteal lymph nodes were removed aseptically and a single cell suspension was prepared in serum free medium. Contamination of macrophages in the cell suspension was removed by allowing them 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 cell suspension

over an adherence 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. The monolayers were again washed and treated with mitomycin-c (1 μg / ml) in complete medium 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 value at 570 nm.

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 (Parajuli *et al*., 1996). Thymocytes obtained from 4-8 week-old C_3H / HeJ 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 mg / 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 standard MTT assay and the values are presented as OD at 570 nm.

Assay for Arginase activity

Arginase activity was assayed by a method as described earlier (Parajuli *et al*., 1996). 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 $ZnSO_4$ 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. 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 / 3×10^5 cells.

Assay of macrophage phagocytic activity

Phagocytosis was determined by the method of Oda and Maeda (Oda *et al*., 1986) with modification. Treated or untreated TAM cultured on glass coverslips kept in 35 mm petridish (Tarson, India) were incubated with heat killed yeast cells (2×10^8 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 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.

% Phagocytosis=No. Of macrophages exhibiting phagocytosis / Total no. macrophages

Assay of pinocytosis

The pinocytic activity of macrophages was measured according to the method described earlier (Gupta et al.,1988) TAM were treated with 50 μg / 0.5 ml of horse radish peroxidase (HRP) solution in complete medium for 1 h. The cells were then washed with PBS and lysed in 0.05% triton-x 100. 2.5 ml of assay buffer (6 ml 0.1 N Sodium Phosphate buffer pH 5.0, 0.60 ml of 0.3% H_2O_2, 0.05 ml of 10 μg / ml O-dianisidin) was added to 0.4 ml of cell lysate. Reaction mixture was kept at 37°C for 1 h and the optical density was measured at 460 nm.

Assay of TNF activity

The activity of TNF in the culture supernatant of TAM was measured by dye uptake assay as described earlier (Kumar *et al*., 1994). 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:

% Cytotoxicity = C-T / C X100

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.

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 time.

RESULTS

Effect of *in vivo* administration of thymus extract on the cell count of TAM

In this experiment we checked if *in vivo* administration of thymus extract could increase the number of TAM. Thymus extract was administered to tumor-bearing mice at a dose of 5 mg / kg body weight (a dose found to be optimum in preliminary experiments), in multiple doses, each being administered on the 4th, 6th, and 8th days after DL transplantation and the TAM were isolated on the 10th day. Total numbers of adherent cells were counted as described in materials and methods. Results are shown in Fig, 1. *In vivo* administration of thymus extract resulted in an increased count of TAM as compared to PBS administered mice.

Effect of *in vivo* administration of thymus extract on the phagocytotic activity of TAM

In this experiment we checked if *in vivo* administration of thymus extract could enhanced the phagocytic activity of TAM. Thymus extract was administered to tumor-bearing mice at a dose of 2.5 mg / kg body weight, 5 mg / kg body weight and 10 mg / kg body weight, in multiple doses, each being administered on the 4th, 6th, and 8th days after DL transplantation and the TAM were isolated on the 10th day, phagocytotic activity was studied after treatment *in vitro* with LPS (10 µg / ml). As shown in Table 1. TAM obtained from tumor- bearing mice administered with thymus extract at a dose of 5 mg / kg body weight 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. Treatment of these TAM *in vitro* with LPS resulted in a further increase of the phagocytotic activity.

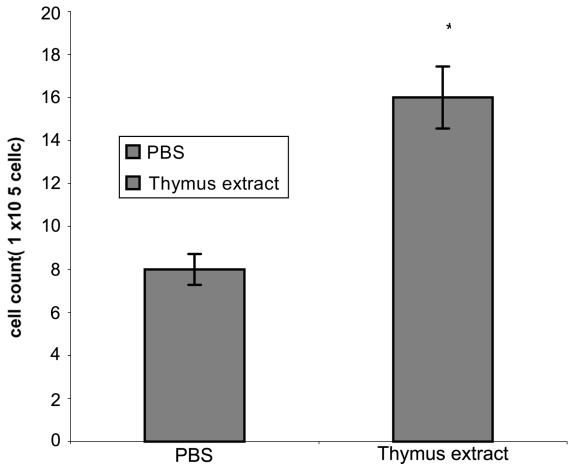

Fig. 1. TAM were obtained from mice administered with thymus extract in multiple doses of (5 mg / kg body weight) each being administered on the 4th, 6th and 8th day after DL transplantation. Number of adherent cells was counted as described in materials and methodsValues are mean±SD from a representative experiment done in triplicate. In other experiments similar results were obtained. *p<0.05 vs values of TAM obtained from mice administered with PBS.

Table 1. Effect of *in vivo* administration of thymus extract on the phagocytotic activity of TAM

In vivo treatment	PBS	Thymus extract (5 mg / kg B.wt)
Medium	25±1.9	70±6.8*
LPS	36±3.1	150±11#

TAM obtained from DL-bearing mice administered with thymus extract or PBS were incubated *in vitro* in medium alone or containing LPS (10 mg / ml) for 24h. Yeast cells 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. #p<0.05 vs. values for corresponding control of TAM incubated *in vitro* in medium with LPS.

Table 2. Effect of *in vivo* administration of thymus extract on the pinocytic activity of TAM

HRP uptake (Absorbance at 460 nm)		
In vitro treatments	PBS	Thymus extract
Medium	0.165±0.01	0.177±0.09
LPS	0.216±0.02*	0.284±0.04*

TAM obtained from DL-bearing mice administered with thymus extract or PBS were incubated *In vitro* in medium alone or containing LPS (10 μg / ml) for 24h. Pinocytic activity was assayed as described in materials and methods.
*p<0.05 vs values for corresponding control of TAM incubated *in vitro* in medium without LPS.

Effect of *in vivo* administration of thymus extract on the pinocytic activity of TAM

TAM were obtained as described for results of table1 from mice administered with PBS, or thymus extract in multiple doses and the pinocytic ability of these TAM was studied after treatment *in vitro* with LPS (10 μg / ml) as described in materials and methods. Results are shown in Table 2. TAM obtained from tumor- bearing mice administered with thymus extract showed a significantly higher pinocytic activity as compared to TAM obtained from mice administered with PBS.

Effect of *in vivo* administration of multiple doses of thymus extract on antigen presenting ability of TAM

TAM were obtained as described for results of Table 1 from mice administered with PBS, or thymus extract in multiple doses and the antigen presenting ability of these TAM was studied after treatment *in vitro* with LPS (10 μg / ml) as described in materials and methods. Results are shown in Fig. 1. TAM obtained from DL-bearing mice administered with thymus extract showed a significantly higher antigen presenting ability as compared to TAM obtained from mice administered with PBS alone on incubation *in vitro* in medium without LPS. *In vitro* LPS treatment of TAM obtained from mice administered with thymus extract resulted in further augmentation of the antigen presenting ability.

Effect of *in vivo* administration of multiple doses of thymus extract on the IL-1 production by TAM

Multiple doses of thymus extract were administered to DL-bearing mice as described for results shown in Table 1 and the TAM were harvested on the 10 th day. These TAM were incubated *in vitro* for 24 h in the presence or absence of LPS (10 μg / ml) and supernatant were checked for IL-1 activity. Results are shown in Table 3. TAM isolated from DL-bearing mice administered with thymus extract showed significant IL-1 activity, even in the absence of LPS treatment in vitro. Treatment of these TAM in vitro with LPS resulted in further augmentation of IL-1 production.

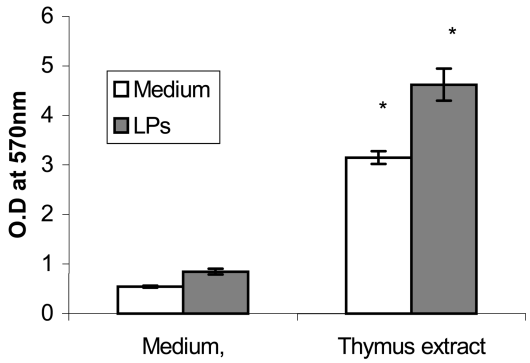

Fig. 2. TAM were incubated for 24h in medium alone or containing the thymus extract (5 mg / kg body weight) and the antigen presenting ability was studied. Values are mean±SD from a representative experiment done in triplicate. In other experiments similar results were obtained. *p<0.05 vs values for TAM incubated in medium without LPS. $^{\$}p$<0.05 vs values for corresponding control of TAM incubated in medium without LPS and thymus extract. $^{\#}p$<0.05 vs values for TAM incubated in medium with LPS and without thymus extract.

Table 3. Effect of *in vivo* administration of thymus extract on IL-1 production by TAM

Thymocytes proliferation (OD at 570 nm)		
In vitro treatment	PBS	Thymus extract
Medium	2.14±0.11	3.68±0.57
LPS	3.419±0.6	4.035±0.4*

TAM obtained from DL-bearing mice administered with thymus extract in multiple doses as described for Table 1, were incubated in vitro in medium alone or containing LPS (10 μg / ml) for 2 4h. Cell-free culture supernatants were harvested and assayed for soluble IL-1 activity. 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 of TAM incubated *in vitro* in medium without LPS.

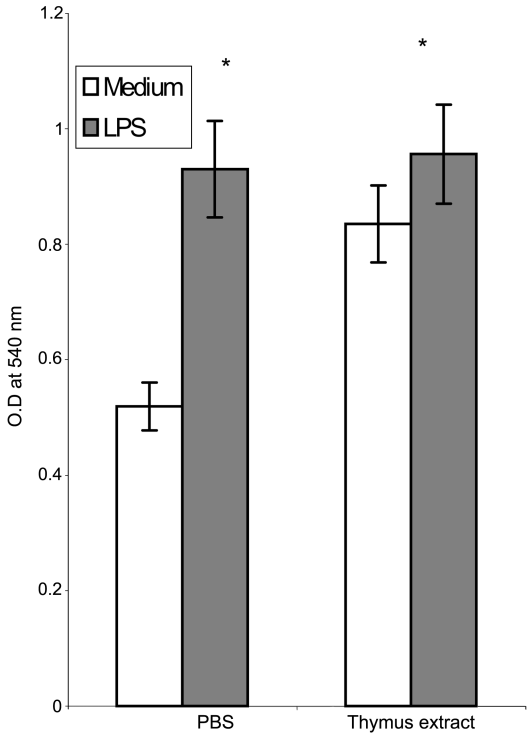

Fig. 3. TAM were incubated for 24h in medium alone or containing the thymus extract (5 mg/kg body weight) and the arginase activity was studied. Values are mean±SD from a representative experiment done in triplicate. In other experiments similar results were obtained. *p<0.05 vs values for TAM incubated in medium without LPS.

Effect of *in vivo* administration of thymus extract on the arginase activity of TAM

Next we investigated the effect of *in vivo* administration of thymus extract or on the arginase activity of TAM. TAM obtained from PBS or thymus extract administered mice were incubated for 24h in the presence or absence of LPS (10 μg/ml) and the supernatant were assayed for arginase activity. Results are shown in Fig. 3. TAM obtained from mice administered with *thymus* extract exhibited arginase activity, which was further, augmented on LPS treatment.

Effect of *in vivo* administration of thymus extract on the production of TNF by TAM

TAM obtained from PBS or thymus extract administered mice were incubated for 24h in the presence or absence of LPS (10 μg/ml) and the supernatant were assayed for production of TNF, results as shown in Table 4. TAM isolated from

Table 4. Production of TNF by thymus extract or PBS treated TAM

% Cytotoxicity against actinomycin-D treated L929 cells±S.D.		
In vitro treatments	PBS	Thymus extract
Medium	5±0.4	20.9±2.0*#
LPS	10±1.4*	36.7±3.5*#

TAM obtained from DL-bearing mice administered with thymus extract or PBS were incubated *In vitro* in medium alone or containing LPS (10 μg/ml) for 24 h. The culture supernatant Were harvested and assayed for TNF production as described in materials and methods.
*p<0.05 vs values for corresponding control of TAM incubated *in vitro* in medium without LPS. #p<0.05 vs values for corresponding control of TAM incubated *in vitro* in medium with LPS

DL-bearing mice administered with thymus extract showed significant level 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.

DISCUSSION

We have previously reported a wide range of immunosuppressive actions on the progressive growth of DL (Parajuli *et al*., 1996; Parajuli *et al*., 1997). Further, it was suggested that the inhibition of TAM mediated tumor cytolysis involved, among several possibilities, suppressor molecule secreted /induced by tumor cells (Kumar *et al*., 1994). In order to reverse the tumor growth associated inhibitory effect on TAM, the aim of the present study was to investigate if thymus extract could augment the inhibited response of TAM in the DL-bearing host.

It was observed that intra peritoneal administration of thymus extract to a DL-bearing mice resulted in an increase in number of TAM aspirated from the ascitic fluid, Furthermore augmentation of the phagocytic ability was also observed. Phagocytosis is one of the basic and fundamental function of macrophage to clear dead cell debris and non self components . Phagocytosis is also the one of the primary step in the process of antigen uptake by macrophage leading to antigen presentation (Barker *et al*., 2002). Indeed, we observed that the

antigen presenting ability of TAM obtained from mice administered with thymus extract was enhanced. Although the mechanisms of the reversal of tumor growth associated effects on TAM upon administration of thymus extract are not clear but some of the possibilities can be considered. One of the actions of thymus extract could be direct on macrophage activation. Indeed, we show that thymus extract not only enhanced LPS responsiveness of TAM but could also activate TAM even in the absence of LPS stimulation *in vitro*. These observations indicate that the requisite signal transduction required for the optimal activation of TAM can be achieved by *in vivo* administration of thymus extract as such with no scope left for any further activation of the tumoricidal functions on *in vitro* treatment with second activation signal of LPS. The mechanism(s) of such augmentation, however, remains unclear but some of the possibilities can be considered. It has been reported earlier that administration of thymic peptides results in an enhanced production of IFN-g (Baumann *et al*., 1997) which is a potent macrophage activating cytokine (Adams *et al*., 1984) and a decrease in the production of PGE_2 which is inhibitory to macrophage activation (Alleva *et al*., 1994; Evans *et al*., 1983) However, we have shown earlier that PGE_2 is not involved in DL-mediated suppression of TAM activity (Parajuli *et al*., 1997). Therefore, it is suggested that one of the mechanisms of TAM activation could be through the enhanced production of IFN-g. Indeed, unpublished observation of our laboratory showed that intraperitoneal administration of thymic peptide to DL bearing mice resulted in an enhanced level of IFN-g (Shanker *et al*., 1999). Moreover, it is also likely that the optimal cytotoxic activation of TAM consequent to *in vivo* administration of thymus extract could be the result of a synergistic action of various peptides that constitute the thymus extract. Indeed, thymus extracts are reported to contain peptides like thya1 and Prothymosina, which have been reported to show a wide range of immunomodulatory potential (Eckert *et al*., 1997). However, activation of TAM upon *in vivo* administration of thymus extract did not clarify if TAM were directly responsive to these peptides. Results of the experiments where TAM obtained from untreated DL-bearing mice were treated *in vitro* with thymus extract confirmed that TAM were directly responsive to thymus extract for activation and thymus extract 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 (Onozaki *et al*., 1985), which is a co-stimulatory signal for lymphocyte proliferation indeed due to antigen presentation by macrophages and TNF, which is found to activate monocyte and macrophages as well as to mediate their cytotoxic activity (Adams *et al*., 1984; Kumar *et al*., 1994). The mechanism of the enhanced antigen presenting ability of TAM upon treatment with thyα1 could also be correlated to the production of IL-1 and TNF, which have potential for autocrine stimulation of macrophages. This cytokine has been shown to enhance the antigen presenting ability of macrophages and to stimulate them to express high levels of MHC-II molecules, which are necessary for antigen presenting activity (Adams *et al*., 1984).

It has been demonstrated that tumor rejection or growth is associated with fate of arginase metabolism through NO synthase or arginase pathways in TAM (Mills *et al*., 1991). Although we showed an up-regulation of IL-1and TNF production but a simultaneous increase in arginase activity was not observed upon in vivo administration of thymus extract. 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 (Parajuli *et al*., 1996). However, the observation showing upregulation of IL-1, and TNF but not of arginase activity upon administration indicate that thymus extract interferes with tumor growth associated “switching on” of the arginase pathway. TNF is found to activate monocyte and macrophages as well as to mediate their cytotoxic activity (Kumar *et al*., 1994). Further experiments will be necessary to understand the mechanism of the activation of TAM by thymus extract. An augmentation of monocyte activity by thymic peptides in cancer patients has been reported but the mechanism of such activation remained unclear (Eckert *et al*., 1997). Expression of receptor(s) for thymic peptides on monocyte/

macrophage is not proven as yet and even the second messenger mechanism has remained elusive (Garaci *et al*., 1985). Therefore, the possibility of TAM getting activated through an indirect mechanism via the enhanced production of other cytokines resulting in their synergistic action on macrophages is not ruled out. Our results are also supported by the reports where tumoristatic activity and TNF secretion of monocyte from tumor-bearing hosts were fully restored by full thymus extract but only partially by individual thymic peptide (Garbin *et al*., 1995). Taken together the results of the present investigation suggest, for the first time that thymus extract can be used to fully overcome the tumor-induced state of unresponsiveness of TAM for activation to tumoricidal state. As such TAM have been reported to be resistant to activation by various immunomodulators, the present study therefore suggests that thymus extract could be used for immunotherapeutic purpose in a tumor-bearing host to exploit the tumoricidal potential of TAM.

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