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Activation of cell mediated immune response and apoptosis towards malignant cells with turmeric treatment in murine model

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The effect of ethanolic turmeric extract (ETE) on murine lymphocytes vis-à-vis tumor cells was studied, in terms of its ability to activate lymphocytes and to induce apoptosis in tumor cells. Degree of activation and proliferation of lymphocytes treated with ETE was analyzed in terms of blastogenesis, DNA synthesis through ^{3}H-thymidine incorporation and cell cycle analysis by flourescence activated cell sorter (FACS). FACS analysis was also carried out to observe the proliferation as well as apoptosis of tumor cells. Morphological condition of both the cell types in presence of ETE was examined by scanning electron microscopy (SEM). Cytotoxic capability of ETE-treated effector T lymphocytes towards tumor cells was judged *in vitro* by ^{51}Cr-release assay and the growth of tumor *in situ*. ETE stimulated murine lymphocytes towards blastogenesis and synthesis of DNA, as revealed by increased incorporation of ^{3}H-thymidine. FACS indicated that the lymphocytes were driven towards mitotic cycle by activating G_2-M transition. In the same count, the tumor cells mostly remained accumulated in the G_2-M phase, and thus mitotically arrested. Scanning electron photomicrographs revealed the blastoid transformation of lymphocytes and ETE-induced apoptotic condition of tumor cells. Furthermore, ETE-treated T cells were cytotoxic towards tumor cells *in vitro*, as shown by ^{51}Cr- release assay. ETE administered intravenously or orally could delay the onset and growth of tumor, and thus prolonged the life span of the tumor-bearing host. The present investigation suggests potential of turmeric both to destroy the malignant cells directly and via activation of the host's cellular immunity.

Keywords: Turmeric, Lymphocytes, Immunostimulatory, Tumor cells, Apoptotic

Turmeric (the rhizome of *Curcuma longa* L.) has been widely used as a yellow coloring agent and spice in many foods, and in indigenous medicine for the treatment of inflammatory and other disorders. It has been considered as anti-bacterial[1-2], anti-viral[3-5], anti-protozoan[6] and a strong antioxidant[7-12]. It inhibits proliferation of a wide variety of transformed cells such as HeLa[13], Jurkat[14], prostrate cancer[15], MCF-7[16], and AK-5 tumor cells[17]. Curcumin, a diferuloyl methane is the major pigment in turmeric. Several studies have also shown the inhibitory effect of turmeric or curcumin in different experimental tumorigenic models[18] and it has been found to be a potent inhibitor for the initiation and promotion of tumors in animals with chemical carcinogen, such as 12-*O*-tetradecanoyl-phorbol-13 acetate (TPA)[19], 1,2-dimethylhydrazine dihydrochloride (DMH)[20], dimethyl benathracene (DMBA)[21], benzo[a]pyrene[22], 7,12-dimethylbenz[a]anthracene[23] etc.

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Although extensive work has been carried out on inhibitory activities of turmeric and curcumin towards tumor growth, carcinogens, mutagens and viruses, not much attention has been paid to the effect of turmeric on lymphocytes. In the present investigation, we have studied the effect of ethanolic turmeric extract (ETE) both on lymphocytes vis-à-vis tumor cells. The efficacy of ETE as activating agent for lymphocytes has been assessed by the degree of blastogenesis and DNA synthesis i*n vitro*. Progression of the activated lymphocytes in cell cycle has been studied with the aid of fluorescent activated cell sorter (FACS), which in addition to the measurement of DNA content per cell analyzes the percentage of cells at different stages of mitotic cycle in a given population. FACS also reveals the quantum of cells arrested in mitotic pathway and likely to proceed towards apoptosis[24, 25]. Morphology of both lymphocytes as well as tumor cells treated with ETE has been studied through scanning electron microscopy. Earlier, we have shown that with activation, cytotoxic T lymphocytes (CTLs) are differentiated to a killer state, capable of destroying tumor target cells[26, 27]. In the present study,

the cytotoxic activity of CTLs has been judged by ^{51}Cr-release assay after ETE treatment. The effect of ETE, administered intravenously or orally on the solid tumor growth *in situ* has also been presented.

Material and Methods

Animals

Inbred adult Swiss mice of both sexes, 8-12 weeks of age were used for all experiments. Breeding nuclei were obtained from Indian Institute of Chemical Biology, Kolkata and were maintained with food and water *ad libitum* in our animal house.

Tumor induction

Ehlrich ascitic carcinoma cell lines were obtained from Chittaranjan National Cancer Research Institute, Kolkata and maintained in our laboratory by serial passages.

Maintenance of ascitic tumor cell line

Tumor cells were collected from peritoneal exudates of the mice bearing ascitic tumor by aspiration with a syringe fitted with 27-gauge needle. They were then washed twice by centrifugation for 5 min at 1000 rpm with cold sterile phosphate buffered saline (PBS) and resuspended in PBS at a concentration of 10^6 cells in 0.1 ml PBS and injected intraperitoneally to the mouse for induction of ascitic tumor. The full-grown ascitic tumor developed within 10 to 15 days. After every 20 days the ascitic tumor cells were transferred to new mice following the same protocol as a routine for serial passage. Average life span of ascitic tumor bearing mice was 28 ± 4 days.

Solid tumor induction

To induce a solid tumor, 10^6 tumor cells, suspended in 0.1 ml PBS were injected subcutaneously at the base of thigh of left leg of a normal mouse. The mean time for appearance of palpable tumor at the site was 14 days.

Ethanolic turmeric extract (ETE) preparation and dose

Fresh rhizomes of turmeric (*Curcuma longa* L.) were obtained from the local market. After cleaning properly with water, 10 g of sample was crushed to a paste with mortar and pestle and 10 ml absolute alcohol was added to the paste and kept in a refrigerator at 4°C for overnight. The extract was then filtered through Whatman filter paper 1 and the filtrate was refiltered again through cellulose acetate Millipore filter paper (0.2 μm porosity, Sartorius) for sterilization. The final solution obtained was aliquoted and stored at 4°C. To ascertain the dry weight of ETE, a volume of ETE in each batch was evaporated to dryness under reduced pressure (Rotary Vaccum, EYELA, Japan) at 55°C. The average dry weight of ETE was 0.435 ± 0.032 mg/ml. As suggested by our earlier studies[28-29], 25 μl dose of ETE was used in the present study. The equivalent amount of ethanol (25 μl) was used for control, and this protocol was maintained for all the experiments.

Separation of T lymphocytes

Spleen and lymph node cell suspensions in 3 ml of PBS were layered on Ficoll and Hypaque solution (Type IV, Sigma Co., USA) and centrifuged at 3000 rpm for 10 min. The band of lymphocytes at the junction of Ficoll Hypaque and PBS was taken out by Pauster pipette and washed twice with PBS. The ficoll-hypaque purified lymphocytes were finally resuspended in prewarmed RPMI 1640 supplemented with 10% goat serum[30] and the lymphocyte preparation was poured on a nylon wool fiber column for separation of T cells[31]. For preparing the column, 0.1 g teased and sterilized nylon wool (Robins' Scientific Corporation, USA), soaked in RPMI was gently packed in a 1 ml syringe. The columns were loaded with cell suspensions (6×10^6 lymphocytes in 1 ml) and incubated at 37°C for 1 h. Non-adherent T cells were eluted out with an excess amount of warm RPMI and re-suspended in fresh medium. T cells were counted with the help of haemocytometer.

In vitro cell culture medium

The cells were suspended in minimum essential medium (MEM, Hi-Media) supplemented with glutamine, HEPES buffer, 200 mg $NaHCO_3$/100 ml, 100 U of penicillin/ml, 100 μg/ml streptomycin, 50 μg/ml nystatin and 10% heat-inactivated sterile goat serum.

Measure of *in vitro* blastogenesis

The transformation of T lymphocytes into blasts after *in vitro* activation by ETE was studied. *In vitro* blastogenesis of lymphocytes was carried out in glass culture tubes (Borosil) of volume 5 ml with a density of 10^6cells in 2 ml of culture medium. The percentage of blast was enumerated with an occulometer fitted on eyepiece of the microscope, in presence of trypan blue. Cells with diameter over 6 μm were considered as blast.

Measure of DNA synthesis

Blast transformation is usually accompanied by DNA synthesis and cell proliferation. DNA synthesis at different intervals of turmeric treatment was measured by incorporation of ^{3}H-thymidine (^{3}H-TdR) into DNA. Cells were obtained from mice injected (i.v.) earlier with 25 µl of ETE and suspended at a concentration of 2×10^6 cells/ml in culture medium (RPMI-1640), of which 200 µl of cell suspension was aliquoted in each well of a 96-well micro-culture plate. 1 µCi of ^{3}H-thymidine (sp. act. 18.5 Ci/Mm, Bhaba Atomic Research Centre, Mumbai) was added to each well The micro-culture plate was incubated for 8 h at 37°C in humidified atmosphere containing 5% CO_2 in air. At the end of the culture period, cells were harvested with a PHD cell harvester (Cambridge, MA) on to glass fiber filters, washed with methanol, dried and kept in standard scintillation vials (Beckman, USA). At the time of radioactivity count, 5 ml of scintillation fluid (6 g PPO, 0.5 g POPOP/L of toluene) was added into each vial. Radioactivity was counted in β-scintillation counter (LS 1800 Beckman, USA). All assays were done in triplicate and the level of ^{3}H-TdR incorporation was expressed as counts per min.

Cell cycle analysis by FACS

For cell cycle analysis, normal as well as ascitic tumor bearing mice were injected intravenously with 25 µl of ETE. After 24 h, suspensions of spleen cells and ascitic tumor cells were prepared in PBS separately. To 1 ml of cell suspension, 1 ml of 80% ethanol was added and the cells were fixed overnight at 4°C. Fixed cells were centrifuged at 1000 rpm, the supernatant was decanted off, and 0.5 µl of 500 µg/ml (standard 250 µg/ml) RNAse A was added, followed by incubation for 45 min at 37°C. The cells were centrifuged at 1000 rpm and suspended in 0.5 ml of 69 mM ethidium bromide at room temperature for 30 min (69 mM ethidium bromide was prepared in 38 mM sodium citrate). Ethidium bromide is a fluorochrome, which stains nuclear DNA. Finally, the cell cycle analysis was done in fluorescence activated cell sorter (FACS, Caliber, Becton Dickinson).

Scanning electron microscopy (SEM)

Lymphocytes and tumor cells were collected separately from mice, injected intravenously with 25 µl of turmeric extract for 24 h. The cells were fixed in Karnovsky fixative for 3 to 4 h at 4°C. A drop of cell suspension was taken on clean glass stubs (approx 18 × 18 mm) and waited for 5-10 min to allow the cells to settle down and were air dried. The cells were washed in cocodylate buffer twice for 10 min, dehydrated with an ascending grade of acetone (30-50-70-80-90-95% twice for 10 min each) at room temperature and finally kept in dry acetone. Thereafter, the cells were dried by critical point drying method, substituting dry acetone from the cells by CO_2. After drying, cells were coated with gold in a fine coat ion sputter (J.C.F. 1100) by mounting the glass stub containing the cells on a brass stub with electro-conducting paints. Cells were then examined and photographed under scanning electron microscope (Leo 435 VP) at AIIMS, New Delhi.

^{51}Cr release assay (Cytotoxicity assay)

Cytotoxic ability of T lymphocytes was determined by using ^{51}Cr release assay[32]. This assay is based on the fact that radioactive chromium ions ($^{51}Cr_3O_4$) diffusing into a cell are retained in the cytoplasm for a considerable period of time. This internal ^{51}Cr is released into supernatant fluid; following cell membrane damage caused by cell mediated cytotoxic response of the effector lymphocytes. In this study, 10^7 tumor target cells (Ehlrich ascitic carcinoma cells) in 1 ml suspension labeled with 200 µCi sodium chromate (Na_2 $^{51}CrO_4$, sp. act. 50 mCi/mg, BRIT, Mumbai) by incubating for 90 min at 37°C in humidified atmosphere containing 5% CO_2 in air. The tube containing the cells was shaken thrice during incubation for proper labeling and after incubation, the cells were washed three times with PBS and the number of cells was adjusted to 1×10^3 cells in 0.25 ml. These radioactive chromium labeled cells were used as target cells for cytotoxic assay.

Effector T lymphocytes were collected from mice treated earlier with 25 µl of ETE for 48 h. To 1 ml of effector cells, 250 µl of ^{51}Cr labeled target cell suspension was added in three different target: effector ratios (1:100, 1:50 and 1:10) and the mixture was incubated for 6 h. The aliquots of 250 µl containing 10^3 target cells only were taken separately for spontaneous and maximum release. In the tubes for spontaneous release of isotope from labeled target cells, no effector cell was added and instead 1 ml of culture medium was added to keep the volume same with the experimental tubes. In the set of tubes for maximum release of radioactivity from the target cells, 1 ml of distilled water (keeping the volume same with experimental tubes) was added instead of

medium. After 6 h of incubation, culture tubes were centrifuged at 1000 rpm and 1 ml of supernatant was collected from each tube and the amount of ^{51}Cr-released into the supernatant was assessed by using gamma-ray spectrometer (Model no. GR532A, ECIL, India). The percentage of cytotoxicity was calculated in following manner.

$$\% \text{ of Cytotoxicity} = \frac{\text{Experimental-Spontaneous release}}{\text{Maximum release-Spontaneous release}} \times 100$$

Effect of ETE on tumor growth and survivality of hosts

The efficacy of ETE in controlling the growth of tumors was also investigated. For this, the lymphocytes in tumor bearing mice were stimulated by repeated intravenous injections as well as by oral administration with ETE (25 µl) by the following schedules: *Schedule I* — Two doses of ETE were administered intravenously (i.v.) with an interval of 1 week followed by subcutaneous injection of tumor cells (10^6 cells in 0.1 ml of PBS) on the 4th day after 2nd dose of ETE; and *Schedule II* — Oral administration of ETE was done thrice on 0, 3rd and 6th day, and tumor cells were injected subcutaneous on 10th day. In this experiment, three different concentrations of tumor cells $10^6/10^5/10^4$ cells in 0.1 ml of PBS were used. Solid tumor growth of individual mice was measured every 7th day using a slide caliper. Size of a tumor was determined as an average of two readings at right angles by the slide caliper and expressed in cm^2. Each schedule consisted of 6 mice and the experiments were repeated thrice.

Results

Blastogenic response and DNA synthesis of lymphocytes with ETE treatment

The treatment of ETE caused increase in number of blasts over the control (alcohol) with peak at 24 h (Table 1). The kinetics of DNA synthesis was also in agreement with the blastogenesis. At 24 h, the ^{3}H-TdR incorporation was higher in lymphocytes than control (Table 1).

Cell cycle analysis with ETE treatment

FACS analysis also showed the similar trend as in blastogenic response. The percentage of lymphocytes at the G_0-G_1 phase was lower in the ETE group in comparison to the control, suggesting turmeric possibly had driven the cells quickly into the next phase, i.e., S-phase. At G_2-M phase, ETE treated lymphocytes showed a percentage of 12.63 cells in comparison to 3.34 percentage in the control (Table 2 and Fig. 1a & b). This indicated that ETE had driven majority of the lymphocytes towards mitotic cycle. Whereas ETE treatment for 24 h significantly reduced the proliferation rate of tumor cells, resulting in higher percentage of tumor cells at sub G_0-G_1 phase (apoptotic phase), in comparison to the control (Table 2 and Fig. 1c & d). The comparable trend remained for the next G_0-G_1 phase. Accumulation of arrested cells in this phase and decline in the percentage of cells at S-G_2-M phase indicated that tumor cells were arrested from entering the mitotic cycle.

^{51}Cr-release with ETE treatment

So far, it seemed that ETE could activate the murine lymphocytes to a reasonable degree in terms of blastogenesis and DNA synthesis. The FACS analysis also supported the contention of activation of lymphocytes by ETE. So, it became quite necessary to study whether the ETE-mediated activation could drive the T cells all the way to cytotoxic state against tumor cells. ETE-treated T lymphocytes showed significantly higher percentage of cytotoxicity towards tumor target cells than the alcohol-treated and normal lymphocytes with the three different target: effector cell ratios (1:100, 1:50 and 1:10). The cytotoxicity indices with different ratios (Table 3) produced a graded response, indicating ETE-generated effector T cells performed just like the effector cells activated by other means[31-35]

Inhibition of tumor growth and increased survivality of hosts with ETE treatment

Since cytotoxic activation of lymphocytes occurred by ETE treatment, it was necessary to study whether

Table 1—Blastogenic responses and pattern of incorporation of ^{3}H-thymidine by the lymphocytes treated for different intervals with ETE and ethyl alcohol (25 µl)

[Values expressed as mean ± SD, Two-way ANOVA revealed all the treatment values were significant compared to control ($p < 0.01$)]

	Percentage of blasts			DNA synthesis (CPM)		
	24 h	48 h	72 h	24 h	48 h	72 h
ETE-treated	18.97 ± 0.76	14.78 ± 1.41	11.93 ± 1.06	73691.06 ± 705	23892.9 ± 305	10717.6 ± 593
Alcohol-treated	2.87 ± 2.69	3.46 ± 2.82	2.03 ± 2.73	3479.8 ± 407	4931.6 ± 201	2261.6 ± 424

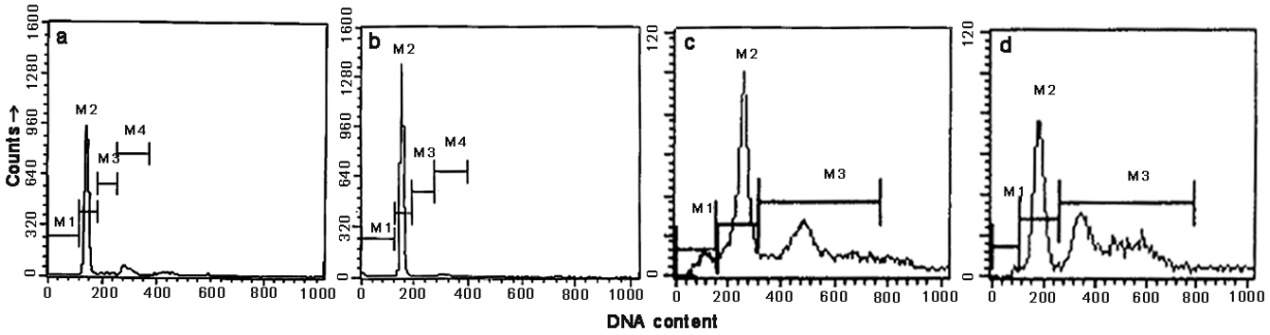

Fig. 1—Cell cycle analysis by FACS on the basis of percentage of the lymphocytes at different stages after 24 h treatment **a**) with 25 µl ETE and **b**) 25 µl alcohol (control) [Cell cycle stages: M1 represents sub G_0-G_1; M2, G_0-G_1; M3, S; M4, G_2-M]. FACS analysis of Ehlrich ascitic carcinoma cells after 24 h treatment with **c**) 25 µl ETE and **d**) same amount of alcohol (control) M1 represents sub G_0-G_1; M2, G_0-G_1; M3, S-G_2-M]

Table 2—FACS analyses indicating percentage of cells at different stages of cell cycle after 24 h treatment with ETE and alcohol (control)

Cell cycle stages (Peak)	Lymphocytes (%)		Ehlrich ascitic carcinoma (%)	
	ETE-treated	Control	ETE-treated	Control
Sub G_0-G_1 (*M1*)	0.96	2.36	5.04	0.30
G_0-G_1 (*M2*)	67.83	73.88	47.84	38.34
S (*M3*)	2.39	1.25	41.80	57.80
G_2-M (*M4*)	12.63	3.34	(S-G_2-M phase under M3 peak)	(S-G_2-M phase under M3 peak)

Table 3—Percentage of cytotoxic response mounted by ETE-treated lymphocytes towards tumor target cells at different target: effector cells ratios

[Values expressed as mean ± SD. Two-way ANOVA revealed all the treatment values were significant compared to control (p <0.01)]

	Target: Effector cell ratio		
	(1:100)	(1:50)	(1:10)
ETE-treated	93.24 ± 3.45	70.84 ± 2.36	64.27 ± 2.76
Alcohol-treated	58.06 ± 4.67	41.76 ± 2.5	38.71 ± 5.05
Normal	53.86 ± 3.98	39.21 ± 4.45	33.28 ± 4.12

the treatment *in vivo* could restrict the tumor growth. The rate of tumor development was relatively slower in both the schedules of administration of ETE than the control mice, as evident from Figs 2 and 3. The figures also indicated the increase in survivality of the tumor bearing hosts with ETE treatment, which was possibly due to slower rate of tumor growth in all the cases. The tumors were palpable, but not measurable upto 4th week in mice receiving ETE intravenously; the survivality of these mice were up to 20th week

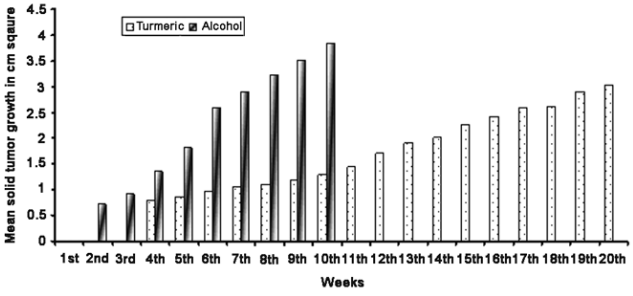

Fig. 2—Effect of 25 µl ETE or alcohol on growth of solid tumor induced with 10^6 cells [ETE or alcohol was administered intravenously twice with an interval of 1 week; on 4th day from 2nd i.v. injection tumor cells injected subcutaneously (as per schedule I in 'Materials and Methods'). Measurement of tumor was stated on every 7th day]

(Fig. 2). However, when ETE was administered orally, tumors became palpable on 3rd week and measurable from 5th week and the tumor bearing mice survived up to 18th week (Fig. 3a). In both cases, 10^6 tumor cells were used for induction of tumor. In two sets of experiment for oral administration, lower concentrations (10^5 and 10^4) of tumor cells were used for induction of solid tumor (Fig. 3b & c). The lower concentrations could induce tumor with some delay, the tumor size did not reach to maximum of 3 cm^2 and survivality of mice increased.

Scanning electron microscopic study

Scanning electron micrograph (SEM) of murine lymphocytes after 24 h *in vivo* ETE treatment did not show any detrimental changes in the cell surface topography (Fig. 4a). On the other hand, SEM images of Ehlrich ascitic carcinoma cells treated with ETE for 24 h revealed disintegration of plasma membrane (Fig. 4b).

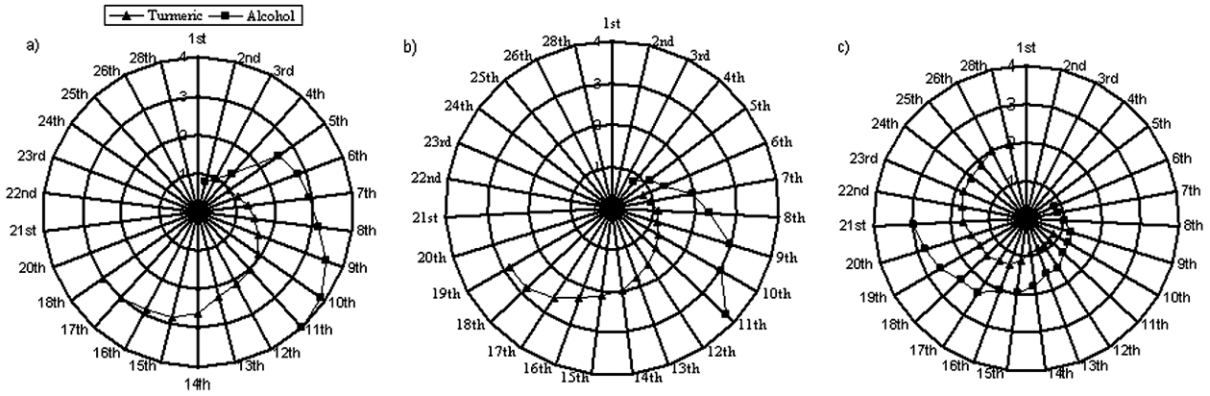

Fig. 3—Rate of solid tumor growth in mice administered ETE orally thrice (on 0, 3rd and 6th day) before tumor induction [Growth rate was measured every week marked clockwise on the circumference. Each concentric circle denotes 1 cm^2 growth indicated by 1 to 4. Three concentrations of tumor cells for induction of tumor were: (**a**) 10^6; (**b**) 10^5 and (**c**) 10^4 cells in 0.1 ml PBS]

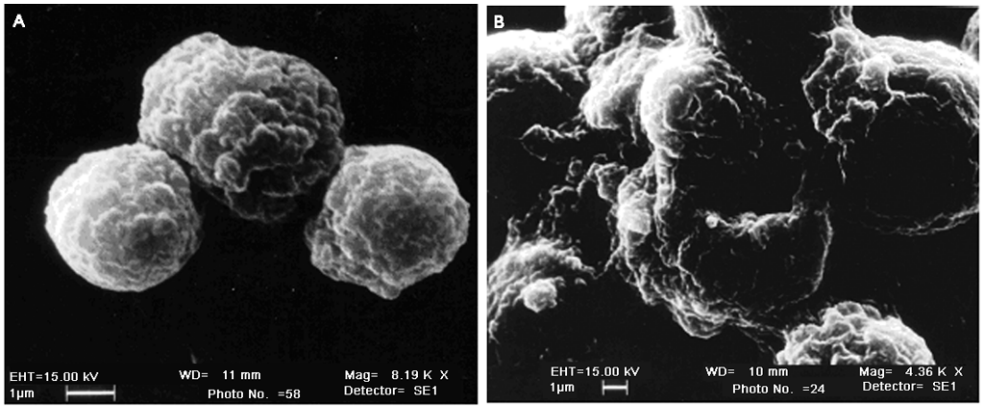

Fig. 4—SEM images of cells after 24 h of *in vivo* ETE treatment (25 µl): **A**) lymphocytes, **B**) Ehlrich ascitic carcinoma cells [Lymphocytes were with healthy look, whereas disintegration of plasma membrane of tumor cells could be noted]

Discussion

Treatment with ETE could activate blastogenesis and DNA synthesis of lymphocytes at appreciable levels (Table 1). FACS analysis clearly showed activation of cell cycle with ETE treatment, G_2-M phase transition was notable (Table 2 and Fig. 1a). At the same time, ETE-treated lymphocytes seemed to be in good shape as revealed by scanning electron microscopy (Fig. 4a). Whereas tumor cells with ETE treatment remaind arrested at S-G_2-M phase, it did not allow the cells to enter into the mitotic cycle. Simultaneously, the percentage of cells was higher at the apoptotic phase i.e., sub G_O-G_1 phase (Table 2 and Fig. 1c). Interestingly, disintegration of plasma membrane of these cells was revealed in SEM micrographs (Fig. 4b). Thus, ETE at the same dose (25 µl) regime qualifies it as stimulatory for lymphocytes and apoptotic for tumor cells. The dual effect of ETE on two types of cells are significant findings in this study. This immunostimulatory nature of ETE drives the T cells to become cytotoxic towards tumor cells as judged by ^{51}Cr-release assay *in vitro*. Although the percentage of ^{51}Cr-release was reasonably high in the control groups; a specific reason to which could not be assigned at present, the level of cytotoxicity with ETE was notably higher to recognize potential of ETE in cell-mediated immunity.

Tumoricidal effect of ETE was judged *in vivo* after intravenous (Fig. 2) and as well as oral administration (Fig. 3). ETE was found effective in delaying the appearance and growth of tumor and thus contributing to the longer life span of the tumor-bearing host. On oral administration, ETE was less effective in restricting solid tumor growth than given intravenously, probably due to poor absorption of curcumin through the gastrointestinal tract[33]. Curcumin is biotransformed in the intestinal tract of

humans and rodents and its systemic availability becomes poor[33].

Several workers have suggested the mechanism for inhibition of tumor growth by curcumin. Curcumin has been shown to inhibit tumor growth through the serine/threonine protein kinase C pathway[34], whereas another study[35] suggests inhibition through protein tyrosine kinase transduction pathways. There are indications that the antiproliferative property of curcumin is partly mediated through inhibition of c-myc, c-jun, c-fos mRNA expression and even bcl-2 mRNA expression[36-38]. In an earlier study, we observed a strong antioxidant role of turmeric in inhibiting generation of O_2^- and OH^- and also, notable inhibition of lipid peroxidation in the lymphocytes[7]. This might have reflection in maintenance of integrity of cell membrane, particularly in the lymphocytes, as revealed in SEM images.

In conclusion, ETE at the same dose (25 µl) regime qualifies itself as stimulatory for lymphocytes and apoptotic for tumor cells. Interestingly, the present study demonstrates that turmeric acts as a double-edged sword, stimulating lymphocytes to mount cytotoxic response towards tumor cells and inducing apoptosis in tumor cells directly. We suggest the use of turmeric as immunotherapeutic agent after surgery and radiotherapy, when tumor cell load is minimal, but relapse of tumor growth remains.

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