MATERIALS AND METHODS

ANIMALS

Inbred adult Swiss mice of both sexes, 8-14 weeks of age, were used for all experiments. Breeding nuclei were obtained from Indian Institute of Chemical Biology, Calcutta and are maintained with food and water *ad libitum* in our animal house by inbreeding for many generations.

TUMOR INDUCTION

Ehlrich ascitic carcinoma and Ascitic fibrosarcoma cell lines were obtained from Chittaranjan National Cancer Research Institute, Calcutta and maintained in our laboratory by serial passages. These two lines have been used for the initial experiments, the results were comparable, and we continued with Ehlrich ascitic carcinoma cells, for further experiments.

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. After centrifugation tumor cells were washed twice with cold sterile PBS and resuspended in PBS at a concentration of 10^6 cells in 0.1ml PBS and injected intraperitoneally to the mouse for induction of ascitic tumor. Within 10 to 15 days full grown ascitic tumor develops. Serial passage was carried out after every 20 days. Average life spans of ascitic tumor bearing mice are 28 ± 4 days.

SOLID TUMOR INDUCTION

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

ETHANOL TURMERIC EXTRACT (ETE) PREPARATION

Fresh rhizomes of turmeric *(Curcuma longa* L.*)* were obtained from local market. After cleaning properly with water; 10 gms of sample was crushed to a paste with mortar and pestle and 10 ml of absolute alcohol was added to this paste and kept in a refrigerator at 4° C for overnight. The alcoholic turmeric extract was then filtered through Whatman filter paper 1; the filtrate was refiltered again through cellulose acetate Millipore filter paper (0.2 µm porosity, Sartorius) for sterilization, and the final solution obtained was aliquoted and stored at 4° C in ml

The total amount of ETE prepared during each batch was evaporated to dryness under reduced pressure (Rotary Vaccum, EYELA, Japan) at 55^{0} C to determine the percentage yields of the extract.

DOSES AND ROUTE OF ADMINISTRATION OF TURMERIC EXTRACT

Different doses of turmeric extract 10, 15, 20, 25, 50 and 100µl have been used for *in vitro* viability assay both for lymphocytes and tumor cells. The dose in which lymphocytes survive well but tumor cells become moribund was used for experiments. For *in vivo* experiments, turmeric extract was injected intravenously in lateral tail vein of mice.

As the turmeric extract was made in ethyl alcohol, the equivalent amount of ethanol for particular dose of turmeric was used for control, and this protocol was maintained for all the experiments.

SERUM COLLECTION

Goat (*Capra bengalensis*) serum was used for *in vitro* supplementation of culture medium. This serum was found to be effective as Fetal Calf Serum (FCS) for *in vitro* culture of lymphocytes (Chaudhuri & Chakravarty, 1983). Goat blood was collected aseptically from

jugular vein and was allowed to stand at room temperature for 45 mins for clotting and then at 4^0C for the next three hours. Serum was gently collected and then centrifuged at 1000 rpm for ten minutes. The clear serum was preserved in aliquots at -20^0C until use. When required, the serum was thawed, heat inactivated at 56^0C for 45 mins and sterilized by passing through Millipore filters (0.45 μm, Sartorius).

SEPARATION OF MACROPHAGES (MφS)

Spleen and lymph node were collected aseptically from mice and the cells were dissociated in phosphate buffered saline (PBS, pH 7.2) with the help of stainless steel wire mesh; further dissociation of the cells was done by passing through a syringe fitted with 27-gauge needle. It was then transferred to sterile plastic petri plates and incubated at 37^0 C for 45 mins in humidified atmosphere of 5% CO_2 in air. After incubation freely suspended non-adherent cells (mostly lymphocytes) were collected in a centrifuge tube for further separation of B and T cell, while the adherent macrophages (Mφs) remained attached to the petridish surface. The petri dish surface was given three mild flushes of chilled PBS with Pasteur pipette to obtain the Mφs.

SEPARATION OF B AND T CELLS

The non-adherent cell types, depleted of Mφs, were washed in PBS by centrifugation at 1500 rpm for 5 mins, and resuspended in RPMI 1640 with 10% goat serum.

In another approach Ficoll and Hypaque gradient was employed for the separation of lymphocytes from the total cell suspension. Spleen and lymph node cell suspensions in 3ml of PBS were layered on Ficoll and Hypaque solution (Type IV, Sigma Co., USA) and centrifuged at 3000 rpm for 10 mins. The band of lymphocytes at the junction of Ficoll Hypaque and PBS was taken out and washed twice with PBS. The ficoll-hypaque purified lymphocytes were finally resuspended in prewarmed RPMI 1640 with 10% goat serum. Then

the lymphocyte preparation was poured on a nylon wool fiber column for separation of B and T cells, as outlined by Julius and co-worker (1973).

For preparing the column 0.1gm teased and sterilized nylon wool (Robins' Scientific Corporation, USA), soaked in RPMI was gently packed in a 1ml syringe. The columns were loaded with cell suspensions (6 X 10^6 lymphocytes in 1 ml) and incubated at 37^0C for 1 hr. Non adherent T cells were eluted out with an excess amount of warm RPMI and re-suspended in fresh medium. The column was then filled up with chilled RPMI and further incubated in ice for 10 mins. Thus Nylon wool adherent B cells were eluted out with an excess amount of cold RPMI by agitation of the wool and then re-suspended in fresh medium. T and B cells were counted with the help of haemocytometer.

IN VITRO CELL CULTURE MEDIUM

The cells were suspended in Minimum Essential Medium (MEM, Hi-Media, Mumbai) or RPMI 1640 supplemented with glutamine, HEPES buffer, 200 mg $NaHCO_3$/100 ml, 100 U of penicillin/ml, 100 mg/ml streptomycin, 50 mg/ml nystatin and 10% heat inactivated sterile goat serum.

IMMUNIZATION

Sheep's erythrocytes were used as model antigen for immunization. Blood from jugular vein of a sheep was collected in Alsiever's solution and then two washes were made with PBS. The final pellet was diluted on volume to volume basis with PBS to make 25% of Sheep RBC (SRBC). For primary immunization 0.1ml and for secondary immunization 0.05 ml of the 25% SRBC were injected in mice intravenously in lateral tail vein.

IN VITRO VIABILITY ASSAY

Different doses of ethanolic turmeric extract were added to splenic lymphocytes, ascitic fibrosarcoma cells and Ehlrich ascitic carcinoma cells in glass culture tubes (Borosil) of

volume 5ml at a density of 1×10^6 cells in 2 ml of culture medium. Cell survival at different hours (4, 6, 8, 12, 16, 24, 48 and 72 hr) of culture was judged by trypan blue dye exclusion test. Counting of the cells was made with haemocytometer.

LYMPHOCYTE PROLIFERATION ASSAY

Lymphocyte proliferation during primary and secondary immune responses to SRBC was judged after intravenous injection of turmeric, one day prior to primary immunization. Number of viable B and T lymphocytes and also Mϕs from the spleen of the immunized mice were counted by trypan blue dye exclusion test. Simultaneously the weight of spleen was also taken into account.

STUDY OF ANTIBODY MEDIATED PRIMARY AND SECONDARY IMMUNE RESPONSE

Antibody mediated immune response has been measured in different ways:

Plaque Formation Cell Assay (PFC)

Antibody response can be quantified in terms of the number of antibody secreting cells in the haemolytic plaque assay following Cunningham and Szenberg's (1968) PFC method with some minor modifications (Chakraborty and Chakravarty 1983). Suspension of spleen cell from a mouse, immunized with SRBC in 0.1 ml of PBS was mixed with 50µl of SRBC and 50µl of rabbit complement. After thorough mixing with the micropipette, the mixture was transferred into the micro-chambers made by two slides, fixed face to face with a bigummed tape (3M, Minnesota Mining and Mfg. Co., USA). Two slides of the chambers were sealed by dipping in a molten mixture of paraffin and petroleum jelly at 50^0 C. After 8 hrs of incubation of the slides at 37^0 C, typical plaques of lysed RBCs surrounding antibody secreting cells develop and they were counted under binocular microscope with transmitted light.

Haemagglutination Assay

The kinetics of primary and secondary humoral immune responses can easily be measured by the antibody titer technique. Agglutination reactions produce visible clumps of cells or particles bearing antigenic epitopes and antibody directed to them.

For haemagglutination assay, the first well contains 1:10 dilution of the immunized and heat inactivated serum and second well onward two fold serial dilution (1:20 to 1:20480) was made with saline in a 96 well microtitre plate. The first well contained 0.18 ml of PBS and 0.02 ml of serum. An amount of 0.02 ml of 1% SRBC as antigen was added to each well. The microtitre plate was kept at 37^{0} C overnight in humidified atmosphere. Different degrees of haemagglutination of SRBC occurred with antibody titers down the row of wells. Reciprocal value of the serum dilution in the last well giving positive haemagglutination reaction has been considered as agglutination titer value. On the right of this well the absence of haemagglutination revealed as precipitation of SRBCs at the bottom of the well as a red tiny button.

Haemagglutination reaction in presence of 2- merceptoethanol (ME)

2- mercaptoethanol (ME) reduces IgM molecules, but IgG molecules are resistant to this compound and can agglutinate erythrocytes even in the presence of this compound. Thus, whether Ab response was of primary(IgM) or secondary (IgG) nature was determined by adding 5µl of 0.1M 2-ME in a series of wells.

Immunoglobulin G (IgG) estimation by ELISA

Mouse IgG was assayed on microplates precoated with 100µl of serum samples diluted in coating buffer (8µl of serum sample and 1992µl of coating buffer) and incubated overnight at 4^{0}C. After incubation, wells were aspirated and washed 3 times with wash buffer (300µl/well). Then the plates were blocked with 200µl of 10% bovine serum albumin (BSA) and kept at room temperature for 1 hr. After incubation, plates were washed 4 times with wash buffer and then 100µl of standards (10pg/ml), controls and rabbit anti-mouse IgG primary antibody (GeneI, Bangalore) was pipetted into the wells and incubated at room temperature for 2hrs. Again the wells were washed 4 times with wash buffer and horseradish

peroxidase linked polyclonal goat anti-rabbit IgG (Genel, Bangalore) was added to the wells and incubated for 1 hr. Following a wash cycle to remove any unbound antibody-enzyme reagent, a substrate solution (hydrogen peroxide and tetramethyl- benzidine) was added to the wells and incubated for 30 mins. The enzyme reaction yielded a blue product that turned yellow on addition of stop solution (1 M H_3PO_4). The optical density of the plate was read at 450 nm in an ELISA reader (Biotech, MIOS). Readings of the different dilutions of standard mouse IgG were used for determining the concentration of IgG on the basis of Beer Lambarts' Law.

MEASURE OF *IN VITRO* BLASTOGENESIS

The transformation of T and B cells into blasts after *in vitro* activation by ethanolic turmeric extract was studied. Blastogenic activity of turmeric was also compared with that of Concanavalin A (Con A), a polyclonal activator. Con A type IV (Sigma Chem. Co., USA) at a concentration of 5μg/ml was used for blastogenic transformation of murine T cells (Chakravarty and Chaudhury, 1981, 1983; Chakravarty and Maitra, 1990). The percentage of blast was enumerated with a haemocytometer 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 hours 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 turmeric extract and suspended at a concentration of 2 x 10^6 cells/ml in culture medium (RPMI-1640), of which 200μl of cell suspension was aliquot in each well of a 96-well micro-culture plate. The micro-culture plate was incubated for 8 h at 37° C in humidified atmosphere containing 5% CO_2 in air in the presence of 1 μCi of ^{3}H-thymidine (Sp. Act. 18.5 Ci/Mm, Bhaba Atomic Research Centre, Mumbai) per well. At the end of the culture

period, cells were harvested with a PHD Cell Harvester (Cambridge, MA) onto 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 gm PPO, 0.5 gm POPOP/lt 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 minute.

CELL CYCLE ANALYSIS BY FACS

For cell cycle analysis, mice were injected intravenously with turmeric extract and at different intervals (16 and 24h), spleen cell and ascitic tumor cell suspensions 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^{0} C. Fixed cells were centrifuged, the supernatant was decanted off, and 0.5μl of 500 μg/ml (Standard 250 μg/ml) RNAse A was added, followed by incubation for 45 mins at 37^{0}C. 69 mM ethidium bromide was prepared in 38 mM sodium citrate. The cells were centrifuged and suspended in 0.5 ml of 69 mM ethidium bromide (a fluorochrome which stains nuclear DNA) at room temperature for 30 min. Finally the cell cycle analysis was done in fluorescence activated cell sorter (FACS, Caliber, Becton Dickinson), in which a laser beam and light detector were used to count the DNA content of single intact cells in suspension. The peak in the DNA histogram refers to percentage of cells at a particular stage of cell cycle.

SCANNING ELECTRON MICROSCOPY (SEM)

Cells treated with turmeric extract *in vitro* and *in vivo* were fixed in Karnovsky fixative for 3 to 4 hrs at 4^{0}C. A drop of cell suspension was taken on clean glass stubs (approximately 18x18 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 mins. The cells were then 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. After dehydration the cells were dried by critical point drying method, substituting dry acetone from the cells by carbon dioxide. After drying, cells were coated with gold in a fine coat ion sputter (J.C.F. 1100) by mounting the glass stub using 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.

TRANSMISSION ELECTRON MICROSCOPY (TEM)

For Transmission Electron Microscopy cells treated with turmeric extract *in vitro* and *in vivo* were fixed in karnovsky fixative for 2 hrs and then washed with 0.1 M PBS three times at 4^0C. Post fixation was done with 1% osmium tetraoxide for 2 hrs and the cells were again washed with 0.1 M PBS three times. The cells were then dehydrated with an ascending grade of acetone (30-50-70-80-90-95% twice for 10 mins at 4^0C and finally kept in dry acetone for 30 mins room temperature. After dehydration, cleaning was done with toluene twice for 30 mins, followed by infiltration with embedding medium and toluene. After this cells were embedded in pure embedding medium containing araldite (CY212), dodecenyl succinic anhydride (DDSA), 2,4,6 tridimethylamino methyl phenol (DMP-30) and dibutyl phthalate. After this, polymerization was done by keeping the blocks at 50^0C for 12 to 14 hrs and then for 24 to 48 hrs at 60^0C. Finally 0.5 to 2μm ultrathin sections were cut with ultramicrotome. These sections were then placed over copper grids. Cells were double stained firstly with uranyl acetate for 10mins at dark, then again with lead citrate for 10 mins. The prepared grids were then photographed under Transmission electron microscope (Philips CM10, Netherlands) at AIIMS, New Delhi.

ESTIMATION OF NUMBER OF CONJUGATES BETWEEN EFFECTOR LYMPHOCYTES AND TUMOR TARGET CELLS AND THE VIABILITY OF TUMOR CELLS.

Ehlrich ascitic carcinoma cells were collected from peritoneum of mice as mentioned earlier. Cell number was adjusted as 10^5 cells in 0.5ml culture medium. The effector T cells were

obtained from mice injected intravenously with 25μl of ethanolic turmeric extract, following the earlier mentioned protocol. Effector T lymphocytes in 0.5 ml were added in two different target : effector ratios (1:5, 1:10) to the fixed number of 10^5 tumor target cells in 0.5ml, and incubated in complete culture medium for 2hrs at 37° C in humidified atmosphere containing 5% CO_2 in air. After 2hrs of incubation, the number of conjugate formed by association of tumor target cell and one or more T effector cells were estimated microscopically under a phase contrast microscope. Sometimes tumor target cells were found with blebbings and also in moribund condition, which were judged by trypan blue dye exclusion test.

^{51}CR- RELEASE ASSAY (CYTOTOXICITY ASSAY)

Cytotoxic ability of T lymphocytes was determined by using ^{51}Cr release assay. This assay is based upon the finding 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 tumor target cells (Ehlrich ascitic carcinoma cells) were pre-labeled by sodium chromate ($Na_2\,^{51}CrO_4$, Sp. Act. 50 mCi/mg, BARC, Bombay). An amount of fluid containing 200 μCi $Na_2\,^{51}CrO_4$ was added in 1ml of tumor cell suspension containing 10^7 cells and were incubated for one and half hour at 37° C in humidified atmosphere containing 5% CO_2 in air. The tubes containing the cells were shaken thrice during incubation for proper labeling. After incubation the cells were washed three times with PBS and the number of cells was adjusted to $1X10^3$ 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 hrs. To 1 ml of effector cells, 250 μl of ^{51}Cr labeled target cell suspension was added in 3 different target: effector ratios (1:100, 1:50 and 1:10) and the mixture was incubated for 6 hours.

The aliquots of 250 μl containing $1X10^3$ 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 cells were added. 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 hrs incubation culture tubes were centrifuged 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 by the effector cells was calculated using the following formula:

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

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 (25μl) by following different schedules:

Schedule I: Two doses of ETE was administered intravenously with an interval of one week followed by subcutaneous injection of tumor cells (10^6cells in 0.1ml of PBS) on the 4th day after 2nd dose of ETE.

Schedule II: Oral administration of ETE was done thrice on 0 day, 3rd day and 6th day, and tumor cells were injected subcutaneous on 10th day. In this experiment three different concentrations of tumor cells were used; 10^6 / 10^5/ 10^4 cells in 0.1ml of PBS.

Schedule III: ETE was administered intravenously twice with a gap of one week; after 7 days

10^6 tumor cells in 0.1ml of PBS were injected intraperitoneally.

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. For growth of ascitic tumor as per schedule III, weight (gm) and girth (cm) of the mice were taken into consideration. Each schedule consisted of 6 mice and the experiments were repeated thrice.

DELAYED TYPE HYPERSENSITIVITY ASSAY WITH 2, 4- DINITROFLUORO BENZENE (DNFB)

The delayed hypersensitivity type reaction depends on overall T- lymphocyte function. Primary sensitization of the mouse was carried out by applying 0.025 ml of 0.0001% DNFB made in acetone in the right foot pad subcutaneously, and then resensitization was made with 0.025 ml of 0.000001% DNFB in the left foot pad on 8th day. Turmeric was administered intravenously 1hr prior to resensitization. The degree of erythema and induration was measured in terms of the diameter of the resensitized paw in cm from the ninth day onward. Size of the reaction spot was measured twice at right angles by a slide caliper and the average was taken as index for the reaction.

DIFFERENTIAL LEUCOCYTE COUNT FROM DTH MICE

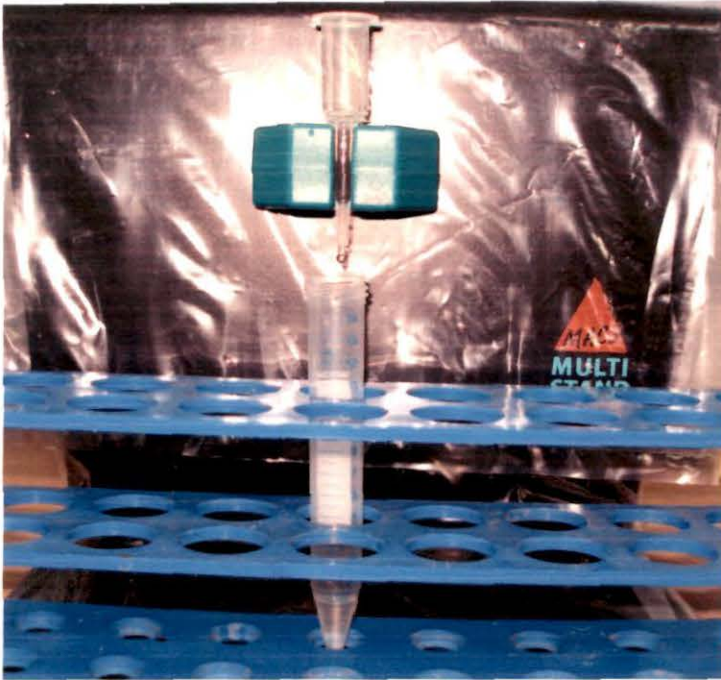
Differential count of the leucocytes at the inflammation site was made, to understand the ratio of WBC in the DTH mice treated with ETE after 24 and 48 hrs of resensitization. For this a thin and uniform blood film was prepared, stained with Leishman's stain and finally counting was done under microscope. The numbers of different types of white blood cells were expressed in percentage.

SEPARATION OF $CD4^+$ HELPER T CELLS FROM THE DTH MICE THROUGH MAGNETIC ASSORTED CELL SORTER (MACS) AND THEIR ENUMERATION

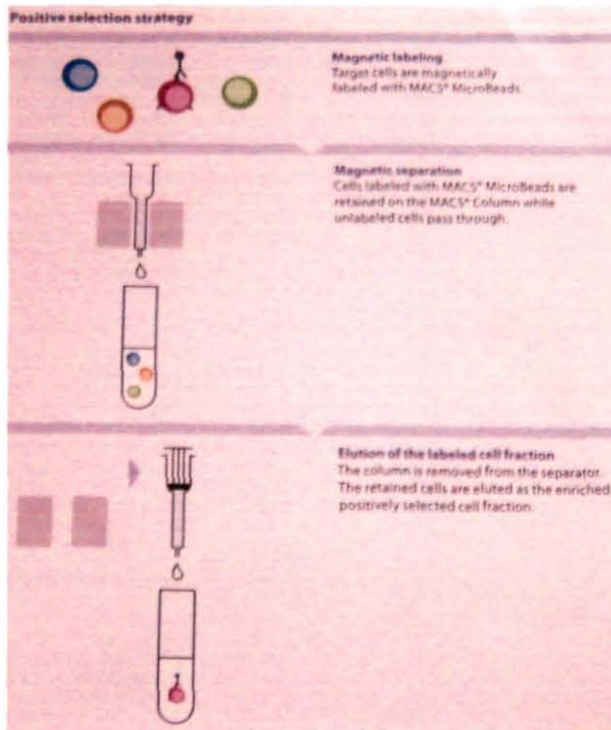
MACS (Mitenyi Biotech, Germany) technology is an extremely efficient magnetic separation method, for cells with specific antigenic markers. With MACS technology, cells of interest were specifically labeled with super paramagnetic MACS microbeads. Then the cells were passed through a MACS column, placed in a strong permanent magnet. The magnetically labeled cells are retained in the column and separated from the unlabeled cells, which passed through the column. After removing the column from the magnetic field, the retained cellular fraction was eluted.

Briefly, lymphocytes isolated from the spleen of DTH mice after 24 and 48 hrs of resentization were suspended in PBS for separating $CD4^+$ helper T cells. Cell suspension was then centrifuged at 1000 rpm for 10 mins and the cell pellet was resuspended at a concentration of 10^7 cells in 80µl of fresh PBS. To this 10^7 cells 20µl of $CD4^+$ (L3TH) microbeads (130-049-201) were added and refrigerated for 15 mins at 4 to 8°C for magnetic separation.

The magnetic separation (MS) column was prepared by rinsing with PBS. 500 µl of PBS was applied on the top of the column to make it wet and finally the effluent was discarded. As soon as the column was prepared the cell suspension containing the microbeads were poured into the column. The unlabeled cells that passed through the column were collected. The column was then washed with 3 times with 500µl of PBS. After collecting the total effluents of the unlabeled cell fractions the MS column was removed from the separator and placed in a suitable collection tube. 1ml of PBS was pipetted onto the MS column and the magnetic labeled cells were immediately flushed out from the column by firmly pushing a plunger into the column. And thus the magnetic labeled cells were collected which were then enumerated by counting them with the help of haemocytometer.



The MiniMACS Separation Unit attached to a MACS Multistand and shown with an MS Column



Digramatical representation of separation strategies with MACS

ESTIMATION OF TNF- α BY ELISA

Solid phase sandwich ELISA KIT (PharMingen, USA) was utilized for the evaluation of TNF-α. Mouse TNF-α was assayed on microplates precoated with affinity purified polyclonal antibody specific for mouse TNF-α. 100μl of standards (ranging from 500pg/ml- 15.6pg/ml), controls and experimental samples was pipetted into the wells and incubated at room temperature for 2hrs. The wells were washed 4 times and horseradish peroxidase linked polyclonal specific for mouse TNF-α was added to the wells. Following a wash cycle to remove any unbound antibody-enzyme reagent, a substrate solution (hydrogen peroxide and tetramethyl- benzidine) was added to the wells. The enzyme reaction yielded a blue product that turned yellow on addition of stop solution. The optical density of the plate was read at 450nm. Concentration of TNF-α was extrapolated from the standard curve of TNF-α (Paul *et al.,* 2001).

BIOCHEMICAL ESTIMATION OF FREE RADICAL

SUPEROXIDE SCAVENGING ASSAY

Superoxide radical (O_2^-) was generated from autoxidation of hematoxylin and was detected by an increasing absorbance at 560 nm wavelength in a UV-visible spectrophotometer (ELICO, S L164). The reaction mixture contained 0.1 M phosphate buffer (pH-7.4), 0.1mM EDTA, 50μM hematoxylin, 25μl of ETE. The final volume of the reaction mixture was adjusted to 2.5ml by adding double distilled water (Martin, Daiby and Sugrman, 1987). The inhibition of autoxidation of hematoxylin in presence of extract over the control was calculated.

HYDROXYL ION GENERATION

Hydroxyl radical was generated from Fe^{2+}-ascorbate-EDTA-H_2O_2 system (Fentons' reaction) which attacks the deoxy D-ribose and a series of reaction that eventually resulted in the

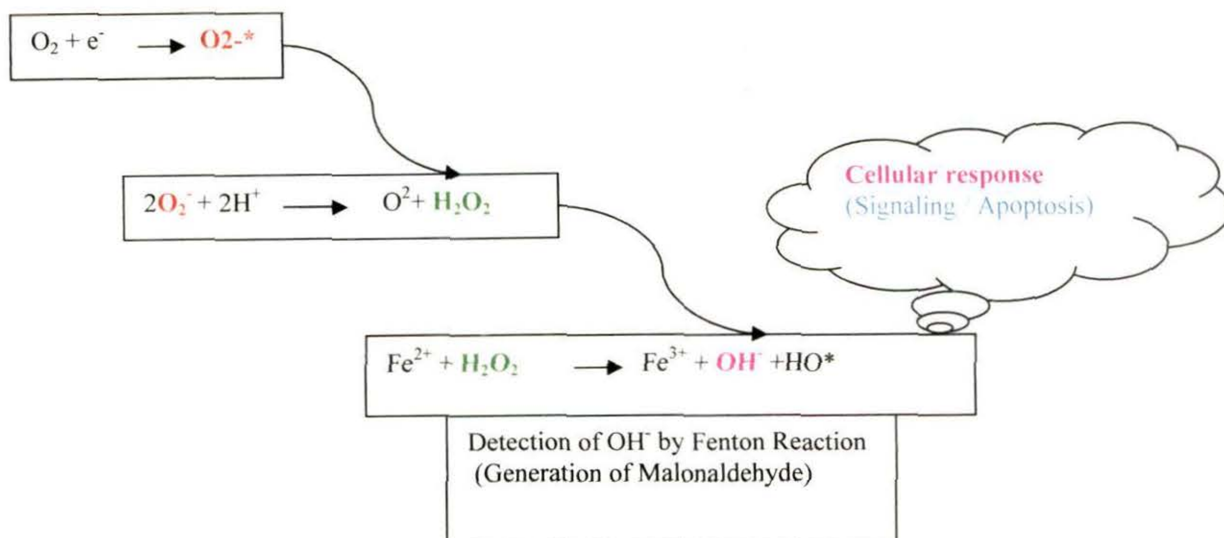
formation of malonedialdehyde (MDA). The reaction mixture contained 2.8 mM 2-deoxy D-ribose, 20mM of $KH_2 PO_4$ - KOH (pH-7.4), 100 mM $FeCl_3$, 104 μM EDTA, 1mM H_2O_2, 1mM ascorbic acid and 25 μl of turmeric extract. The reaction mixture was incubated at 37^0C in humidified atmosphere containing 5% CO_2 in air for 1 hr. Then 2 ml of TBA-TCA reagent was added in each tube and boiled for 15 min. The color of the reaction mixture changes to a pink MDA-TBA chromogen which was finally measured at 532 nm in UV-spectrophotometer (ELICO, S L164). The level of hydroxyl radical generation was expressed as nM of MDA generated/hr (Halliwell, Gutteridge and Aruma, 1987).

Lipid Peroxidation

Lipid peroxidation of lymphocytes and tumor cells with the influence of turmeric extract was estimated separately according to Miller and Aust, 1989. Lipid peroxidation was induced by copper - ascorbate system and estimated as thioburbituric acid reacting substances (TBARS). The thiobarbituric acid assay is the most frequently used method for determining the extent of membrane lipid peroxidation *in vitro*. Malonedialdehyde (MDA), formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of the peroxidation reaction. MDA has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a red species absorbing at 535 nm.

The reaction mixture contained $1X10^6$ packed cells in 0.2M phosphate buffer pH (7.4), with 20mMTris-HCl, 2mM $CuCl_2$, 10mM ascorbic acid and 25μl of ethanolic turmeric extract and were incubated for 1hour at 37^0 C in humidified atmosphere containing 5% CO_2 in air. Lipid peroxidation was measured as malonedialdehyde (MDA) equivalent using trichloroacetic acid (TCA) , thiobarbituric acid (TBA) and HCl (TBA-TCA reagent : 0.375% w/v TBA, 15% w/v TCA and 0.25 N HCl).

After incubation 2ml of TBA-TCA reagent was added and the mixture in each tube was shaken thoroughly. The tubes were then placed in a water bath for 15 mins and then centrifuged for 10 min at 1000 g. Finally the supernatant from each tube was taken turn wise in a cuvette and the OD value was determined spectrophotometrically at 535 nm. Results of lipid peroxidation have been expressed as nanomols of MDA produced/hr/10^6 cells.



NITRIC OXIDE SYNTHASE (NOS) ACTIVITY

NOS activity was determined by measuring the conversion of oxyhemoglobin to methemoglobin according to Jia *et al.*, 1996[35]. L- arginine was found to be the precursor for the synthesis of NO by vascular cells. Cytosolic NAPDH dependent monoxygenase is responsible for the conversion of L-arginine to NO. L–arginine first undergoes monohydroxylation to N^G- hydroxyl-L-arginine which is then oxidized to L-citrulline and produces NO. This NO then undergoes oxidation with oxyhemoglobin (HbO_2) and produces methemoglobin (met Hb). Thus the formation of metHb indicates the production of NO.

Briefly $1x10^6$ packed cells (lymphocytes or tumor cells) were incubated for 2 hr with 50 mM Tris-HCl buffer (pH 7.4), 10mM L-arginine, 64mM hemoglobin, with 25µl of turmeric extract at 37° C in humidified atmosphere containing 5% CO_2 in air. After incubation reaction mixture was centrifuged at 1000rpm for 5 min and the optical density of supernatant was measured in UV- spectrophotometer (ELICO, S L164) at 535nm. Results of NO production were expressed as pmol of NO produced/hr.

To confirm that the production of NO was actually due to the activation of NOS, a competitive inhibitor of nitric oxide synthase (NOS), 10µM N^G methyl- L-arginine acetate ester (NAME) was added in a separate set of experimental tubes.

ISOLATION AND CHARACTERIZATION OF THE DIFFERENT FRACTIONS OF ETE

Crude ethanolic turmeric extract (ETE) was first monitored on a fluorescent bound silica TLC plate (E Merck, Germany) with the solvent mixture containing 1:1 petroleum ether / ethyl acetate. The ethanolic turmeric extract was subjected to chromatographic separation over silica gel (60-120 mesh, SRL, India). Ethanolic turmeric extract was evaporated to dryness under reduced pressure (Rotary Vaccum, EYELA, Japan) at 55^0C. The dried extract was then dissolved in a low polar solvent (Petroleum ether, BP-60 to 80^0C) and was added into the column with the help of a pipette. Finally column space above the adsorbent is filled with solvent with varying ratios of petroleum ether:ethyl acetate. The samples separated were finally collected in different small conical flasks. Fractions were dried under reduced pressure (Rotary Vaccum , EYELA, Japan) at 55^0 C to ascertain their respective dry weight in the ethanolic turmeric extract. The fractions were then dissolved in ethanol keeping the dry weight same as they were present in the ETE for further experimentations.

To characterize the crude extract as well as the fractions eluted, UV spectrophometric analysis was done by UV-Spectrophotometer (Shimadzu, UV-160, Japan).

IMMUNOLOGICAL ASSAYS TO JUDGE THE POTENT FRACTION

The potent fraction was judged through the biological assays mentioned earlier such as *Lymphocyte Proliferation Assay, Study of Antibody Mediated Primary and Secondary Immune Response in terms of antibody-secreting cells by Plaque Forming Cell (PFC) Assay, Hemagglutination assay, Heamagglutination reaction in presence of 2- mercaptoethanol (ME) and Immunoglobulin G (IgG) estimation by ELISA.*

To ascertain the fraction equivalency aforesaid assays were also carried out with different concentrations of commercially available curcumin (ACROS, Belgium) in ethanol (10, 25, 50 and 100μg/ml).

STATISTICAL ANALYSIS

Each experiment was done in triplicate and repeated more than thrice. This was applicable for all experiments. Results are expressed as Mean$\pm$SD of *n* observations Statistical significance was analyzed using students t test as well as using ANOVA software package. Rate of tumor growth was drawn according to the least-square fit method, and the slope for the line was calculated.