MATERIALS AND METHODS

Animals : Male Swiss (albino) mice of six to twelve weeks of age were used in all experiments of the present study. Breeding nuclei were obtained from the Indian Institute of Chemical Biology, Calcutta. They were maintained in our animal colony with pellet food and water ad libitum.

Anaesthetizing the animals : For anaesthetizing, animals were injected intraperitoneally with thiopentone sodium (May and Baker, Bombay) in the dose of 0.07 mg/gm of body weight.

Tumour induction : Chemical carcinogen, 3'-Methylcholanthrene (MCA) was used throughout the study for tumour induction. Histopathological examinations of MCA-induced tumours were always found to be fibrosarcoma in nature. Different doses of MCA in 0.2 ml of Freund's Incomplete Adjuvant (Difco Laboratories, USA) containing mineral oil were injected subcutaneously in anaesthetized mice. Freunds Incomplete Adjuvant was used only to make suspension of MCA. Adjuvant alone does not have any tumour inducing property as revealed from the experiments where 0.2 ml of adjuvant was injected subcutaneously in the animals (unpublished observation).

Tumourogenicity of different doses of MCA was measured in terms of latency period, percentage of appearance of tumour and the life span of the tumour bearing mice (Table 1). The life

span or survival of tumour bearing hosts was the function of tumour growth. So, the rate of tumour growth was also taken into consideration (Fig. 1). Size of the tumour of individual mouse was measured every 7 day by using a slide calliper. Rate of growth of tumours was noted as change in mean diameter (cm^2) of all tumours of living animals at 7 days intervals and mean life span of tumour bearing mice was calculated.

3'-Methylcholanthrene products of two different manufacturers, Koch-Light Laboratories Ltd., Colnbrook, UK and Sigma Chemical Co., St. Louis, USA were tested initially to find out the type to be used in this study. The dose of 2 mg MCA per animal of Sigma make showed maximum appearance in minimum time period and causing shorter life span of the tumour bearing mice (Table 1). The growth rate of the tumour induced with this dose was also faster (Fig. 1). Hence the dose of 2 mg of MCA per animal of Sigma make was selected for tumour induction in all experiments of the present study.

Serum : In our laboratory, goat serum was established to be the cheaper substitute for Fetal Calf Serum (Choudhuri and Chakravarty, 1983). Goat (Capra bengalansis) blood was collected aseptically from jugular vein and was allowed to stand at room temperature for 45 minutes. After clotting of the blood, serum was collected by centrifugation and aliquotes of serum were preserved at -20°C until use. Before use aliquotes were placed in water bath at 56°C for complement inactivation.

Cell suspension : Spleen, mesenteric lymphnode and other lymph nodes (cervical, axillary and inguinal lymph nodes pooled together) were collected from mice aseptically. Cells from these lymphoid organs were dissociated in phosphate buffered saline (PBS, pH 7.0 - 7.2) with the help of stainless steel wire mesh. Peripheral blood was collected in 3.13% sodium citrate solution. Erythrocytes in peripheral blood and spleen cell suspensions were lysed by exposure to tris-buffered 0.83% ammonium chloride solution (pH 7.2) for 10 minutes and finally washed thoroughly with PBS.

For in vitro works, instead of PBS, lymphocytes were resuspended in Minimum Essential Medium (MEM, Hi-Media, Bombay) supplemented with 10% goat serum, 50u/ml of penicillin-streptomycin and 50u/ml of nystatin. Henceforth, if not otherwise indicated, MEM will mean the medium with all these supplementations.

In vivo lymphocyte activation : Concanavalin A (Con A) type IV (Sigma Chem. Co., USA) was dissolved in sterilized distilled water and passed through millipore membrane filter with 0.45 µm porosity before intravenous injection. Four doses of Con A, 10,20,50, and 100 µg in 0.1 ml per animal were injected intravenously. After stipulated period, cell suspensions were prepared from secondary lymphoid organs and peripheral blood and the degree of activation was measured in terms of blastogenic transformation of lymphocytes and the rate of DNA synthesis by lymphocytes.

Blastogenesis : Con A activates the lymphoid cells through a series of events leading to the transformation into blastoid cells and the degree of this blastoid transformation of lymphocytes in secondary lymphoid organs and peripheral blood is often considered as the measure of in vivo activation. Cells with diameter in the range of 7-10 µm were scored as medium sized blasts and with diameter greater than 10 µm as large blasts. The proportion of transformed cells was expressed as percent blast cells by counting the viable medium plus large lymphocytes out of every 100 of total viable lymphocytes on haemocytometer in presence of trypan blue.

The detail of blastogenic response of lymphoid cells induced in vivo by the four doses of Con A (10, 20, 50 and 100 µg/animal) have already been studied earlier in our laboratory (Choudhuri and Chakravarty, 1981; Choudhuri, 1983). A few experiments in this line have been performed initially to denote the point of reference. The results mostly corroborated with the earlier observations. Lymphocytes from spleen, lymphnodes and peripheral blood attained the peak of blastogenic response during 48 to 72 hr of in vivo injection of Con A (Fig 2a,b,c,d). As usual, there was a gradation in blastogenic response with the increment of dose of Con A; response being lower with 10 and 20 µg dose of Con A. Although blastogenesis was maximum with a dose of 100 µg/animal (Fig 2d), the blast cells became mostly vacuolated and exhausted and number of dead cells were very high

in compliance with earlier findings (Choudhuri, 1983). After 48 hr of activation with the dose of 50 µg of Con A a good number of blasts were generated and most of them seemed to be in healthy state without showing any sign of vacuolation or exhaustion. More so, the number of dead cells was also not that high on treatment with this dose. So, this dose of Con A (50 µg/animal) was chosen over other doses to generate in vivo activated lymphocytes for different experiments.

DNA synthesis : Activation of lymphocytes leading to blast cells formation is usually accompanied by DNA synthesis; thus the rate of DNA synthesis during blastogenesis is usually an indicative of the degree of activation.

Degree of activation of in vivo Con A stimulated lymphocytes was measured by the incorporation of tritiated thymidine in DNA at different hours. Suspensions of lymphocytes from spleen and mesenteric lymph node from animals at different hours after intravenous injection of 50 µg of Con A/animal were made following the usual procedure. Then 2 µCi of ^{3}H-thymidine (Sp. act. 15.8 Ci/mM; Bhabha Atomic Research Centre, Bombay) was added in 250 µl of spleen and mesenteric lymph node cell suspension in MEM containing 10^{6} cells; triplicates of each cell type at each hour of assay were maintained. The small glass culture tubes containing the cell suspension were incubated for 8 hr in humidified atmosphere of 7.5% CO_2 in air at 37° C. Incubation was terminated by adding 3 ml of cold PBS in each of the culture tubes and the tubes were then centrifuged. Supernatant

was sucked off the tubes leaving the pellet; 1 ml of PBS was added in each tube and thoroughly mixed with the help of a cyclomixer. Then 1 ml of cold 10% trichlroacetic acid (TCA) solution in distilled water (w/v) was added to each tube and all the tubes were kept in refrigerator for overnight. The TCA precipitate of each tube was then filtered on small filter paper discs (Whatman filter paper No.3) under suction pressure and washed with 10 ml of 10% TCA solution. The filter paper discs were dried and kept in standard scintillation vials for counting by liquid scintillation counter (Packard, USA). An amount of 5 ml scintillation fluid was added to each tube before counting. The scintillation cocktail was prepared by adding 6 gm of PPO, 0.05 gm of POPOP in 1 litre of tolune. Level of incorporation of ^{3}H-TdR was denoted by CPM value.

An appreciable level of incorporation of ^{3}H-TdR by the splenic lymphocytes and lymph node cells was observed by *in vivo* stimulation with 50 µg of Con A/animal and the peak was noted at 72 hr after Con A injection (Fig. 3).

Depletion of T cells by neonatal thymectomy and anti-thymocyte serum treatment : As Con A stimulated T cells have been considered to be effective against tumour cells in this study, a few experiments like activation of lymphocyte population depleted of T cells and effectiveness of this activated lymphocytes in curbing the growth of tumour and in cytotoxicity against tumour target cells needed to be looked into. The T cell population was depleted by neonatal thymectomy of the mice and

by subsequent treatment with anti-thymocyte serum.

Thymectomy : Neonatal mice on the day of birth were chilled on ice and under dissection binocular microscope the two lobes of thymus were removed by suction through a small incision at the thoracic region just above the heart. A flame polished Pasteur pipette fitted in a rubber tube attached to the suction pump was used for the purpose. The incision was stitched with a sterilized silk thread. For warming up, operated animals were placed on a cotton bed under table lamp for sometime and then placed in the cage for proper maternal care.

Raising of anti-thymocyte serum (ATS) : Normal Rabbit Serum (NRS) was collected from the blood of a healthy male rabbit (body weight 650 gm) to be used subsequently to raise anti-thymocyte serum.

Rabbit anti-mouse thymocyte serum (ATS) was raised by injection of the rabbit subcutaneously with mouse thymocytes eight times at 7 days intervals. For the first three injections, 10^8 cells in 1 ml of Hank's Balanced Salt Solution (HBSS, pH 7.2, Hi-Media, Bombay) were used per inoculum. From the fourth injection onward 5×10^7 thymocytes in 1 ml of HBSS were mixed with same volume of Freund's Complete Adjuvant (Difce Laboratories, USA). The rabbit was bled by ear vein puncture 72 hr after 8th injection by adopting the technique followed by Reif and

Allen (1964) and 1966) and Baird et al (1971) in mouse. Serum in aliquotes was preserved in deep freeze (-20°C) until use. Before every use, serum was inactivated for 30 minutes in water bath at 56°C and absorbed with packed (10% of the volume of serum) erythrocytes, liver and kidney cells of normal mouse for 1 hr at 4°C.

The efficacy of raised ATS was expressed as lytic index in Table 2 and it was found to be potent to kill thymocytes and a good number of lymphocytes from mesenteric lymph node.

Treatment with ATS : To remove the residual T cells neonatally thymectomized mice at the age of 6 weeks were given five intravenous injections of 0.1 ml of ATS/animal on consecutive days following the method used in lizard by Fujimoto et al, 1976 and Pitchappan and Muthukkaruppan, 1977.

Depletion of thymus dependant cells from animals following the above technique, resulted a poor blastogenic response (Table 3) when 50 µg of Con A was injected i.v. per animal 10 days after last ATS injection. After 48 hr of injection of Con A, only 16 and 18% blasts in lymphocyte population from spleen and lymph nodes respectively were evidant in contrast to 57 and 54% respectively in normal animals of same age.

Indices were rather comparable to the background level when the animals were not subjected to Con A stimulation.

Assay for angiogenesis and tumour growth in the anterior chamber of eye of mouse : The technique outlined by Folkman and his co-workers (1974, 1976) for grafting tumour piece in the corneal pocket of rabbit eye and adaptation of this technique in case of mouse was modified in the present study where the tumour pieces were grafted in the anterior chamber of eye of mouse instead of intra-corneal pocket. Tumour pieces (approximately 0.5 mm^3, containing about 3 x 10^4 cells) were made from rapidly growing MCA-induced solid fibrosarcoma excised from syngeneic mice. Lymphnode pieces containing polyclonally stimulated lymphocytes were made from mesenteric lymph nodes of mice injected 48 hr earlier with 50 µg of Con A per animal. Nonstimulated lymph node pieces for control experiments were collected from normal mice. All the tissue pieces were made in cold PBS. Animals were anaesthetized following the procedure outlined earlier and an incision of about 1 mm was made with surgical blade (No.11, Swann-Morton, England) on the cornea between pupil and circular limbal vessels. Grafts of following combinations were inserted into anterior chamber of eye with the help of a bent fine tip oral pipette and a small tip malleable spatula and positioned near the pupil by applying gentle pressure on the external surface of the cornea. The eyes were rinsed with 2% streptomycin-penicillin solution. The combination of grafts :

A. Tumour piece alone.

B. Tumour piece plus Con A stimulated lymph node piece.

C. Tumour piece plus normal lymph node piece.

D. Tumour piece plus T cell depleted, Con A activated lymph node piece (obtained from neonatally thymectomized animals depleted of residual T cells by ATS treatment and then activated with 50 µg of Con A in vivo).

Two other sets of control experiments with activated or non-activated lymph node piece were also maintained. Degree of vasodilatation and neovascular proliferative reactions induced by tumour graft alone and in different combinations were denoted by + sign(s) in the table. Photographs of neovascular reactions on cornea of the eyes were taken in Kodacolor (100 ASA) film through dissection microscope (Amplival, C.Z.Instruments India Ltd.) after anaesthetizing the animals.

In vitro incubation of tumour piece with lymphocytes prior to grafting in the anterior eye chamber : The tumour pieces were incubated with activated lymphocytes in experimental set and with normal lymphocytes in control set, prior to grafting them in the anterior eye chamber of mouse. Tumour pieces were obtained following the method described earlier.

Ten tumour pieces of approximately equal size were transferred with the help of an oral pipette in each of the Corning culture tubes (10 ml) containing 2×10^6 lymphocytes in 2 ml of MEM. Lymphocytes were collected from spleen and mesenteric lymph node in PBS and resuspended in MEM. In one of the control sets,

2 ml of MEM instead of cell suspension was added to the tumour pieces. Tubes were incubated in humidified atmosphere in presence of 7.5% CO_2 in air at 37°C for 24 and 48 hr. After termination of incubation, tumour pieces were collected from each tube seperately in cold PBS and washed thoroughly with three changes of PBS. Tumour pieces singly were then grafted in the anterior eye chamber of anaesthetized mice in the manner described above. Neovascular reactions induced by the tumour grafts were enumerated as detailed above.

Measurement ^{3}H-Thymidine incorporation by the tumour pieces incubated in vitro with lymphocytes : To understand the biological activity of the tumour pieces incubated in vitro with activated and non-activated lymphocytes, the radioactive thymidine uptake by the tumour pieces were studied. After incubation with the lymphocytes the tumour pieces were washed thoroughly with cold PBS and then five pieces together were transferred in culture tubes (10 ml, Corning) containing 0.125 ml of MEM. 1 µCi of ^{3}H-TdR was added to each tube and the tubes were incubated in inclined position in humidified atmosphere of 7.5% CO_2 in air at 37°C for 12 hr. At the end, tumour pieces were transferred to scintillation vials after three wash with PBS. 50 λ of methylbenzothonium hydroxide (Sigma Chem. Co., St. Louis, USA) was added to each tube for solubilizing the pieces and kept in dark till counting. The radioactivity incorporated by 5 tumour pieces were measured by liquid scintillation counter.

Repeated injections of Con A in tumour bearing animals : Repeated injections of Con A (50 µg/animal) was made by intravenous route of tail vein after necessary warming up of the animals. In one group of animals, the first injection was made 5 days prior to injection of MCA and the second injection was made 5 days after MCA injection. In another set of experiments, Con A was first injected on the day of detection of palpable tumour in MCA injected mice. Then, second onward, the injections were made at 10 days intervals upto 120 days. Control animals for both sets were injected with same amount of normal saline.

The growth of the tumour in different days on the basis of mean diameter (cm^2) was noted as indicated earlier. At the time of plotting the data, the least square fit method was employed.

Measure of the effects of repeated injections of Con A in normal mice : The effects of repeated intravenous injections of Con A were studied with normal animals of 6 weeks of age, injected 5 times with Con A at 10 days intervals, in reference to the change in body weight of animals, weight of spleen and lymph nodes, total cell count of the secondary lymphoid organs, ability of lymphocytes for blastoid transformation and the histological organisation of lymphocytes in spleen and lymph nodes. In this connection, antibody to Con A in the serum of the animals repeatedly injected with Con A was also tested.

Histological preparation : Spleen and lymph nodes of animals undergoing repeated intravenous injection of Con A were

dissected out on 2nd and 10th day of each stimulations. Fixation of the tissues were made in Bouin's fixative. Passing through the graded alcohol series and clearing in xylene, the tissues were embeded in paraffin. 6 µm thick sections were stained in Delafield's Haematoxylin and Eosin (alcoholic) and viewed through binocular microscope and photographed when necessary.

Detection of anti-Con A antibody by Ouchterlony method : 1.5% agar solution in 0.8% NaCl was poured hot on Laxbrg plastic petre dishes (diameter 5.5 cm) to form a layer of about 2-3 mm thickness. After congelation, 5 holes (2-3 mm diameter) were punched surrounding a central well of 7 mm diameter and the distance employed between the adjacent margins of two wells was usually 6.5 - 8.5 mm. Bottom of the basins were sealed with a minute amount of hot agar (0.3%) in order to avoid any leakage. Con A solution (50 µg/0.1 ml) was poured in the central well.

Blood was drawn by puncture of retro-orbital plexus and serum was collected from normal and experimental animals on 10th day of each of 1st, 3rd and 5th injection. Two fold serial dilutions of serum was made with PBS. In one set, dilutions were from 1/2 to 1/16 and in another, from 1/10 to 1/160th. The serial dilutions of the serum were poured in peripheral wells in clock-wise direction centering around the central well containing Con A solution.

Adoptive transfer of activated lymphocytes at the tumour site : Activated lymphocytes from spleen and lymph nodes of the animals,

injected 48 hr earlier with Con A, were mixed together and injected in three different doses (5×10^5, 10^6 and 2×10^6 cells/animal) at the subcutaneous tumour site. Each inoculum of the cells was made on both the sides of a tumour at 180° apart. Injections were made on every 10th day from the day of detection of palpable tumour and upto 120 days. In control experiments, normal non-activated lymphocytes were injected in the same number as mentioned earlier.

Same technique was also employed in transferring the activated lymphocytes depleted of suppressor T cells. Details of technique to remove suppressor T cells will be outlined later. In this set of experiments, the injections of activated lymphocytes continued beyond 120 days as the life span of tumour bearing mice increased.

Removal of suppressor T cells by cyclophosphamide treatment and subsequent stimulation of the residual cells with Con A :

Technique of cyclophosphamide (Cy) treatment for depletion of suppressor T cells was adopted mainly from the works of Ray and Raychoudhuri (1981). Cyclophosphamide (Sigma Chem. Co., St.Louis, USA) was dissolved in sterile distilled water just before use. Four different doses of Cy, 100, 50, 25, and 12.5 mg/kg of body weight per animal in 0.5 ml of distilled water were injected intraperitonially in different groups of animals. Control animals were injected with only 0.5 ml of distilled water.

To select the effective dose of Cy, the weight of secondary lymphoid organs, index of Con A stimulation in terms of blastogenesis and DNA synthesis after Cy treatment were taken into account. After 48 hr of Cy treatment, the residual cell population from spleen and lymphnodes were subjected to both *in vitro* and *in vivo* Con A stimulation.

For *in vitro* stimulation, the spleen and lymph nodes were harvested aseptically in cold PBS, made free of fatty tissue and the adhering medium on the surface was blotted out with filter paper and weighed on monopan balance. Size of these organs after treatment with different doses of Cy was noted and recorded by photography. Cell suspensions from spleen and lymph nodes were made and percentage of blast cells was recorded following usual technique. Then 10^6 cells adjusted in 0.5 ml of MEM were cultured in triplicates in presence of 5 µg/ml Con A in humidified atmosphere of 7.5% CO_2 in air at 37°C for 48 hr. Parallel control tubes were maintained with cells collected from the animals treated with 0.5 ml of distilled water. At the end of culture, the percentage of blast cells was determined.

For *in vivo* Con A stimulation, 50 µg of Con A/animal was injected intravenously after 48 hr of Cy treatment. On 4th day that is 48 hr after Con A injection, spleen and lymph nodes were removed from the animals for physical measurements and

for determining the percentage of blasts, as determined for in vitro experiment. In addition, level of incorporation of ^{3}H-TdR by the stimulated cells from spleen and lymph nodes was measured following usual procedure indicated earlier. Here, 10^{6} cells in 0.5 ml of MEM were lebelled with 1 µCi of ^{3}H-thymidine for 12 hr in humidified atmosphere of 7.5% CO_2 in air at 37°C.

Cytotoxicity assay : The cytotoxic capability of Con A activated lymphocytes has been measured in ^{51}Cr release assay.

Target cells were obtained from rapidly growing solid fibrosarcoma; tumour pieces were minced in 0.25% trypsin and incubated in a test tube in water bath (37°C) for 45 minutes with occasional shaking. Cells in the supernatant were collected at every 15 minutes interval and finally washed 3 times with PBS. 10^{7} cells in 1 ml of MEM was incubated in water bath (37°C) for 1.5 hr with 200 µCi of $Na_2{}^{51}CrO_4$ (Sp.act. 130-193 mCi/mg, Bhabha Atomic Research Centre, Bombay) and washed thrice with PBS.

An aliquot of 10^{4} radiolabelled tumour target cells in 0.25 ml of MEM was mixed at different ratios (1:5 - 1:200) with effector lymphocytes in 1 ml of MEM in small glass culture tubes and further incubated in humidified atmosphere of 7.5% CO_2 in air at 37°C for 4.5 hr. At the end, 1 ml of supernatant was collected from each tube after centrifugation and the radioacti-

vity released in the supernatant from the tumour target cells was measured in Gamma-ray Scintillation Counter (Electronic Corporation of India Limited, Hyderabad). The percentage of cytotoxicity or ^{51}Cr released by the action of effector cells was calculated as follows :

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

Each index is the mean value of triplicates.

Controls were set with effector cells from normal animals without Con A treatment and from animals depleted of T lymphocytes prior to Con A injection.

Cytotoxic ability of lymphocytes activated in absence of suppressor T cells (by pre-treatment with 25 mg of Cy/kg of body weight/animal) was also studied.

Test for T cell nature of the effector lymphocytes by cytolytic assay : Activated lymphocytes were collected from spleen and lymph nodes seperately from the animals injected 48 hr earlier with 50 µg of Con A. An aliquot of 10^6 activated lymphocytes in 0.1 ml of PBS was added to the tubes containing 0.4 ml of two fold serial dilution (1/2 - 1/2560) of anti-thymocyte serum (ATS) in PBS. 20 λ of guineapig's complement was added to each tube. Guineapig's complement was obtained following the method of Herbert (1978) and absorbed with a

packed (10% of volume of serum) mouse erythrocyte, liver and kidney cells at 4°C for 1 hr 7. The tubes were incubated for 1 hr at 37°C. Incubation was terminated by putting the tubes on ice bath and viability of cells was tested by trypan blue dye exclusion test.

The reduction in percentage of blastoid and small lymphocytes from the spleen and lymph node cell suspension after treatment with serum was calculated as follows :

$$\frac{\text{Initial number of cells added} - \text{Number of cells after treatment with serum}}{\text{Initial number of cells added}} \times 100$$

Cells were either blastoid lymphocytes, when reduction in blast cells was considered or small lymphocytes, when reduction in small cells was considered and serum used was either ATS or NRS.