IMMUNE RESPONSES OF ACTIVATED LYMPHOCYTES TO CHEMICALLY-INDUCED TUMOUR CELLS

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This is to certify that Mr. Uday K. Maitra, M.Sc. worked in my laboratory since August 1979 for his dissertation on the topic "Immune Responses of Activated Lymphocytes to Chemically-Induced Tumour Cells" for fulfilment of the requirements of the Degree of Doctor of Philosophy (Science) of the University of North Bengal.

He is conversant with the techniques and literature citedin the dissertation and carried out the work thoroughly. It seems that the thesis is fit for submission for Ph.D. and he is worthy for award of the degree.

A Chakravarty

(Dr. A. K. Chakravarty)

TO MY FATHER

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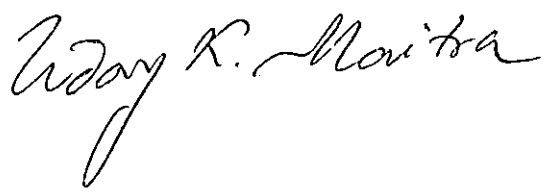

C O N T E N T S

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INTRODUCTION

Specificity of immune responses as expressed in higher animals, have evolved from self non-self recognition mechanism which enable the organisms to escape from foreign invading agents. Foreignness of invading agents is usually attributed to antigenic moities on their cell surface. Acquisition of such foreignness is an usual phenomenon with neoplastic transformation and thus malignant growths are rendered susceptible to the immune response of the organism. Evidences for antigenicity of experimentally-induced tumours were demonostrated by several workers since nineteen fifties (Folly, 1953; Prēhn and Main, 1957; Prēhn, 1960; Klein et al, 1960). Their studies revealed that chemically-induced tumours transplanted to syngeneic animals are rejected, and the rejection was faster when the animals had tumour inoculum after complete excision of primary tumours. This process was considered as manifestation of immunological response induced by antigenic determinants on the tumour cell surface.

Tumour associated antigens (TAA) are broadly categorized in to three classes - oncofetal antigens, antigens of virally-induced tumours and antigens of chemically-induced tumours. Oncofetal antigens are the products of some genes in tumour cells which become silent in the course of embryonic

differention and are re-expressed due to the process of "dedifferention" (Brawn, 1970; Coggin and Anderson, 1974; Alexander, 1975). They serve as individual markers for different tumours and are antigenically poor to elicit any immune response (Baldwin et al, 1972; Fredman et al, 1974; Evans et al, 1979; Mastaka et al, 1984). Sjogren et al (1961), Sjogren (1961) and Appella and Law (1976) observed that antigenic properties of tumours induced by a particular oncovirus share some commonness even when they originate on different tissues or different strain of animals on one hand and on the other different strain of viruses induce tumours of different antigenicity even in the same tissue or in the same strain of animals. This antigenic specificity expressed on tumour cells might be determined by the antigenic constituents of viruses (Habel, 1961) after their genes being integrated with host cell genome (Temin, 1971; Gillespie et al, 1975; Rowson, 1975; Talk, 1977; Zinkernagel, 1979).

Antigenicity of chemically-induced tumours varies from tumour to tumour. The rarity of immunological cross-reactivity amongst chemically-induced tumours arising in the same inbred strain of mice was observed by many workers (Folly, 1953; Klein and Klein, 1962; Old et al, 1962; Old and Boyse, 1964; Bassambrio, 1970; Baldwin et al, 1971b). They pointed out that the variability in antigens was influenced by sex and strain of host, dose and type of carcinogen used and the route of adminis-

tration. By using a single carcinogenic agent Globerson and Feldman (1964) and Wahl et al (1974) could not find commonness in antigens on tumours developed at different tissues of the same animal. This uniqueness of antigens on chemically-induced tumour cell surface could be traced even on the cells isolated from different zones of the same murine fibrosarcoma induced by a single carcinogen (Prehn, 1970). The heterogeneity was also reflected in the differential sensitivity to different drugs which is again related to the distribution of receptors for drugs on the cell membrane (Hapner et al, 1978).

Tumour associated antigens (TAA) are likely to initiate immune responses involving several arms of host's immune system. Involvement of macrophage in tumour immunity has been shown by several workers (Nelson, 1972; Hibbs et al, 1972; Evans, 1976; Horwitz et al, 1979; Taglibaue et al, 1979; Krishnan and Orwell, 1979; Evans and Lawler, 1980; Talmadge et al, 1981). It has been shown that antibody armed or antibody-coated macrophages could destroy the tumour target cell effectively (Tosi and Weiser, 1968; den Otter et al, 1972; Krahenbuhl and Remington, 1974). But in some other reports, no correlation between macrophage content in excised tumour mass and growth of the tumour could be ascertained (Eclas and Alexander, 1974; Loveless and Happner, 1983; Ishi et al, 1984).

Hellstrom and co-workers have shown that tumour bearing host often possesses lymphocytes which are cytotoxic to its

tumour cells in vitro (Hellstrom and Hellstrom, 1969; Hellstrom et al, 1971; Rusell et al, 1976; Vose et al, 1977; Vose, 1980). The number of such lymphocytes declined in hosts having progressive tumours. The cells isolated from solid tumours by Herberman (1974), Betz and Simar (1974); Russell et al (1976) and Lala and Kaizer (1977), were found to be of mixed type mostly Thy-1$^+$ lymphocytes, some IgM$^+$ lymphocytes, null cells and macrophages. However, it has now been accepted that, of all the cell types Thy-1$^+$ or θ^+ cells have the most crucial role in responding against malignancies, since the cytotoxic cells generated in vitro or in vivo against experimental tumours were always found to be Thy-1$^+$ or T lymphocytes (Fredman et al, 1972; Cerottini and Bruner, 1974; Wybran et al, 1974; Ting, 1976; McClusky and Bhan, 1977; Weinstein and Okan, 1980; Herberman et al, 1980; Vose, 1980; Green, 1981; Keder and Weiss, 1981). So employment of T lymphocytes, the chief mediator of tumour immunity, against malignant tumours appeared to be practicable.

Activation of immunity to malignancies by immunization with tumour cells as such is not practical for obvious reasons, like heterogeneity of tumour cell surface antigens, chances of generating some blocking factor(s) in the serum (Alexander, 1974; Sjogren et al, 1971; Hellstrom and Hellstrom, 1969; Hellstrom et al, 1970; Hellstrom and Hellstrom, 1974; Ray and Saha, 1982) and risks of inducing malignancy. Thus other means of

stimulating the host's immune system became imperative.

Mathé and others stimulated the reticuloendothelial system of tumour bearing animals with some non-specific agents like Bacillus Callamette-Guérin (BCG) to control malignant growth and obtained limited success (Mathé et al, 1970; Mathé 1971; Baldwin and Primi, 1971; Zbar et al, 1972; Bartlet et al, 1972; Hawrylko, 1977). Use of Corynebacterium parvum (Woodruff and Boak, 1966; Scot, 1974; Woodruff and Dunber, 1975; Purnell et al, 1976; Prim and Baldwin, 1977; Woodruff and Warner, 1977; Gupta et al, 1978; Meyata et al, 1983) and some other killed micro-rganisms, (Parker et al, 1974; Blast et al, 1975; Pruvell, 1975; Ray et al, 1979; Ray et al, 1980) produced some hopeful results. It had been postulated that these microrganisms mostly stimulate the macrophage arm of immune system. However, T cells are considered to be more effective to combat tumour growth and therefore stimulation of T cells rather than macrophages is likely to inhibit the tumour growth in a much effective way. Again, in view of heterogenous nature of tumour antigens, stimulation of several clones of T cells rather than a specific clone, would be more fruitful. In such a situation, stimulation of T cells in a polyspecific way is possibly more desirable.

Concanavalin A (Con A), a plant lectin and a general mitogenic agent has been shown to activate multiple clones of T lymphocytes in vitro (Stobo and Paul, 1973; Waterfield et al,

1975; Heininger et al, 1976; Chakravarty and Clark, 1977; Green et al, 1978; Chakravarty, 1980). Several of these workers and others (Clark, 1975; Bevan and Cohn, 1975; Bevan et al, 1976; Heininger et al, 1976; Bonavida and Bradly, 1976; Green et al, 1978) have observed that in vitro stimulation with Con A drives the T lymphocytes all the way to differentiate into cytotoxic cells against a variety of targets bearing non-self antigens including tumour cell lines. Moreover, some of the stimulated cells ultimately become memory cells and secondary stimulation with Con A can regenerate the cytotoxic function among these cells as in case of MLC primed cells (Chakravarty and Clark, 1977; Clark et al, 1977; Bonavida, 1977; Chakravarty, 1978).

Anaclerio et al (1974) and Waterfield et al (1976) indicated the possibility of using Con A to activate lymphocytes in the in vivo situations to generate cytotoxic cells. Subsequently, Choudhuri and Chakravarty worked out in detail the process of Con A mediated activation of murine T cells in vivo in terms of blastogenesis, DNA and other macromolecule synthesis (Choudhuri and Chakravarty, 1981; Chakravarty and Choudhuri, 1983) and differentiation of activated cells into cytotoxic cells (Choudhuri, 1983). Thus multiple clones of T cells stimulated simultaneously with Con A in vivo might be effective to mount immune response against chemically-induced malignant cells having diverse type of antigens on their surface. Investigation of such possibility has been the main

objective of the present study.

First, growth and other associated phenomena of tumour explants in the anterior eye chamber of syngeneic animals was studied in the presence of normal and Con A-activated lymphocytes. Anterior eye chamber has long been known as a privileged site for transplantation as, any allograft or heterograft (Gregoir and Symbiose, 1935) and tumour grafts transplanted therein enjoys prolonged survival. Auerbach (1961) used this method for culturing different embryonic tissues of mice in the anterior eye chamber. This technique has been adopted here to co-culture tumour explants with different combinations of activated and non-activated lymphocytes. Moreover, Folkman (1974), Gimbrone et al (1974) and Folkman and Cotran (1976) showed that a piece of malignant tumour when placed in the rabbits' corneal pocket, it induced vaso-proliferations from limbal vessels of cornea towards the tumour grafts. This vaso-proliferation was accompanied by vasodilatations and primary sprouting of capillaries and secondary development of vessels from the tip of the primary vessels. These reactions have nicely been shown to occur with a similar graft in mouse corneal pocket (Muthukkaruppan and Auerbach, 1979). This neo-vascular reaction, known as tumour-induced angiogenesis (TIA) has been shown to be induced by tumour angiogenesis factor (TAF) secreted by the tumour cells (Greenblast and Shubik, 1968; Folkman and Cotran, 1976; Weis et al, 1979; Folkman, 1982). These neovascular reactions were found to precede the growth of almost all the tumour transplants

tested at different sites (Gimbrone and Gullino, 1976; Brem et al (1978; Ziche and Gillino, 1981; Ziche and Gullino, 1982). In this study, the angiogenic reactions and growth of tumour explants grafted in the anterior eye chamber of syngeneic mouse constituted the parameters to measure the effect of activated lymphocytes on tumour explants. In another set of experiments, the tumour explants were incubated with Con A-activated or non-activated lymphocytes in vitro for 24 or 48 hr prior to implantation in the anterior eye chamber.

The efficacy of Con A activated T cells in controlling the growth of tumours was also tested by adoptively transferring at the site of the tumours in hosts.

Next, the lymphocytes in tumour bearing mice were repeatedly stimulated with Con A and the growth rate of tumour and survivality of hosts were studied. Necessary control experiment to study the effects of repeated in vivo stimulation with Con A on lymphoid system was also performed.

Efficacy of Con A generated cytotoxic cells on the tumour target cells was measured in ^{51}Cr release assay. To ascertain that Con A activated T cells are effective in anti-tumour immune response, T cells were depleted by neonatal thymectomy and subsequent treatment of the animals with anti-thymocyte serum prior to stimulation of its residual lymphocytes with Con A.

T cell population is virtually a mixture of functionally different subclasses like effector cells, helper cells and suppressor cells (Gershon et al, 1972; Cantor and Boyse, 1975a, b; Hodes and Hatchecock, 1976; Cantor and Boyse, 1978). Con A being a polyclonal stimulator for T cells, might stimulate all the clones of T lymphocytes including suppressor subset, both in normal and tumour bearing animals (Shou et al, 1976; Raff et al, 1978; Ekstedt, 1979; Eibl et al, 1980; Catalona et al, 1980). It is likely then, that the effector functions of T cells could possibly be augmented if the suppressor subset of T cells can be eliminated from the lymphocyte population.

There are several ways to deplete suppressor cells; the presence of Ly 2,3 markers and the determinants encoded by the I-J subregion of H-2 complex on their surface render them susceptible to alloantiserum against these antigens (Fujimoto et al, 1976; Perry et al, 1974; Perry et al, 1978; Green et al, 1979). They are also sensitive to low doses of radiation (Rotter and Trainer, 1975; Hellstrom and Hellstrom, 1978; Daynes et al, 1979) and the drug cyclophosphamide (Rollinghoff et al, 1977; Tagart, 1977; Green et al, 1979; Ray and Raychoudhuri, 1981; Berd et al, 1982; Berd et al, 1984). The cyclophosphamide sensitivity of this subset of T lymphocytes haa sometimes been employed to enhance the antitumour immune response (Hellstrom, and Hellstrom, 1978; Broder et al, 1978; Yu and McKhann, 1978; Ferguson and Semmons, 1978; Yu et al, 1980; Turk and Parker, 1982; Berd et al, 1982).

In this investigation an attempt has been made to enhance the response of Con A activated lymphocytes by removing the suppressor subset of T cells with cyclophosphamide treatment. The efficacy of suppressor cell depleted, Con A activated lymphocyte population was tesfed by cytotoxicity assay and by adoptively transferring to tumour bearing mice.

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.

RESULTS

ANGIOGENESIS AND TUMOUR GROWTH IN THE ANTERIOR EYE CHAMBER OF SYNGENEIC MICE IN PRESENCE OF CON A ACTIVATED AND NON-ACTIVATED LYMPHOCYTES :

Physiologically active status of a tumour explant in the anterior eye chamber is primarilly indicated by angiogenic reactions like vasodilatation and neo-vascularization from limbal and radial blood vessels of cornea. The distribution of blood vessels in the cornea of a normal mouse could be visualized through dissection bionocular microscope and represented diagrammatically in plate 1 (normal). Within 48 hr of implantation of a tumour piece (Group A), thickening of circular limbal vessel and radiating blood vessels began (Plate 1). By day 4, primary blood capillaries appeared from these dilated vessels in 90% of the Group A animals (Table 4, Fig.1, Plate 2). Secondary capillaries started sprouting from the tips of the primary vessels by day 8 (Fig.2, Plate 2) and by day 12 the number of secondary sprouting increased significantly (Table 4, Fig.3, Plate 2). Majority of the explants capable of inducing secondary vessels gradually grew as a tumour to a point of bursting out of the corneal limit of the eye (Fig.4, Plate 2).

Marked inhibition in angiogenic reactions and growth of the tumour explant were noted when Con A activated lymph node pieces were co-grafted with tumour pieces (Group B). Very feeble initial reactions observed in some of these cases

(Table 4). Which could hardly develop into secondary phase of reactions by day 8 (Fig.2, Plate 3) and only 15% of the tumour explants developed to a perceptible size (Table 4). In this group, 66% of the animals could not develop any secondary capillary sprouting by day 12. But normal non-stimulated lymphocytes could not inhibit angiogenesis and tumour growth in group C animals (Fig.1, Plate 3), rather the reactions were similar to those in group A animals (Table 4).

Control experiments by implantating a normal lymph node piece only and a piece of activated lymph node alone were also performed (Fig. 1,2, Plate 4) and no obvious reaction was observed in both the cases except for slight initial vasodilatation in 30-35% of cases.

The activated lymph node piece from the T cell depleted animals could not inhibit the angiogenesis and tumour growth (Fig.3, Plate 3 and Table 5); rather, both the reactions were similar to those of control group with tumour explant only (Group C Vs A, Table 5). Moreover the degree of tumour growth was higher in this group.

ANGIOGENESIS AND TUMOUR GROWTH INDUCED BY TUMOUR EXPLANTS INCUBATED IN VITRO WITH ACTIVATED AND NON-ACTIVATED LYMPHOCYTES :

As the activated cells could inhibit the angiogenic reactions and growth of tumour in anterior eye chamber, the experiment of in vitro incubation of the tumour explants with

activated lymphocytes and with normal lymphocytes (Control) prior to implantation in the anterior eye chamber were made. The results in table 6 show that in vitro incubation of tumour explants with Con.A activated cells reduced the angiogenesis and tumour growth significantly. Results of tumour explants incubated with normal lymphocytes (Group B, Table 6) were comparable to these of control group (C) where the tumour pieces were not incubated with any cells at all.

INCORPORATION OF ^{3}H-THYMIDINE BY THE CELLS OF TUMOUR EXPLANTS INCUBATED IN VITRO WITH ACTIVATED OR NON-ACTIVATED LYMPHOCYTES :

To test whether Con A activated cells incapacitated some cells of the tumour explant in course of in vitro incubation, the degree of DNA synthesis by the tumour cells in the explants was measured. Maximum incorporation was observed in the case of fresh tumour piece which were not pre-incubated with any cells (Fig.4). Even in this case, the mean radioactivity counts was around 200 which apparently seemed to be pretty low; actually five small pieces of tumour taken together for an index value, had approximately 1.5×10^{5} cells in total (see materials and methods). There was a significant drop (p value $<.05$) in level of incorporation of ^{3}H-TdR when the tumour pieces were incubated with activated lymphocytes for 48 hr (Fig.4). This drop was more with 48 hrs incubation than that with 24 hrs.

STUDY OF IN SITU GROWTH OF TUMOUR AND EFFECT OF ADOPTIVE TRANSFER OF ACTIVATED LYMPHOCYTES AT THE TUMOUR SITE :

When activated lymphocytes, raised in syngeneic animals, were injected at the tumour site, growth of the tumour was at slower pace in comparison to the controls where no cells or non-activated cells were injected (Fig. 5a,6a and 7a). The slopes calculated on the basis of the rate of increase in average size of the tumours in presence of different cell doses indicate that highest cell dose of 2×10^6 cells per animal was most effective (Fig. 7a). The slower rate of growth of the tumours has been reflected in the slower mortality rates of tumour bearing hosts (Fig. 5b,6b,7b). Certain percentage of experimental animals survived up until 150 days, 50 days more beyond the death of control animals (Fig. 7b).

Stimulation of T cells in tumour bearing animals and measure of tumour growth and survivality of hosts :

T cells of tumour bearing mice were stimulated by intravenous injection of concanavalin A following two different schedules :

(i) Two injections of Con A were made, one 5 days prior to the injection of MCA for inducing tumours and another injection given 5 days after MCA injection (Fig. 8).

(ii) Con A was injected at every 10 days after palpable appearance of tumours (Fig. 9) for 12 times.

The slope for the lines indicating the growth rate of tumours (Fig. 8a,9a) clearly show that tumour growth in mice injected with Con A following both the schedules is slower than in controls. However, the slope obtained following the second schedule (0.7, Fig.9a) indicates even slower rate of growth of tumours than the index (1.11) obtained following the first schedule (Fig. 8a). This slower rate of growth in Con A injected mice reflected in the survival rate of the host animals indicated in the inset figures (Fig. 8b,9b).

EFFECTS OF REPEATED IN VIVO STIMULATION WITH CON A ON LYMPHOID SYSTEM OF MOUSE :

Repeated in vivo polyclonal stimulation of lymphoid cells of tumour bearing mice with Con A asks for the study of the effect of repeated stimulation on lymphoid organs and cells and on the host. There is no such report in this area, so, we looked into the following aspects.

a) Measure of body & lymphoid organs' weight and the number of viable lymphocytes in lymphoid organs and in peripheral blood : It was observed that these indices remained almost unchanged (Table 7) during the period of repeated in vivo stimulation with Con A. The lymphocyte concentration per ml of peripheral circulation did not show much change either. Repeated stimulation could not produce notable adverse effect on the general state of health of the animals; as revealed from the average body weight of experimental animals and normal animals (foot

note, Table 7).

b) Blastogenesis : Repeated in vivo stimulation at every 10 day for 5 times could not impair the potentiality of lymphocytes in secondary lymphoid organs and in peripheral blood of being activated to transform into blasts. With each injection of Con A, the blastogenic peak was found by 48 hrs (Fig.10). The number of blasts at peaks were not usually below 40% which was evident even after the last injection done. The percentage of blasts ~~injection of Con~~ A usually came almost to background level after 10 days of each injection of Con A.

c) Histological architecture of lymphoid organs before and after every stimulation : Histological preparations of spleen and lymph node were made 48 hr after each Con A injection that is at the peak hour of blastogenic response and 10 days after each stimulation when the blastogenic response was in wane. The white pulp area is usually dispersed in normal spleen (Fig. 1,2, plate 6). With each stimulation of Con A, distinct germinal centres with conglomeration of dividing lymphocytes were observed (Fig. 3,4, Plate 6). Organization of the germinal centres was similar to that induced with allogeneic stimulation. In normal lymph nodes, the lymphocytes are usually dispersed throughout the organ (Fig. 1,2, Plate 8) with a very narrow marginal sinus beneath the capsule. With Con A stimulation, germinal centre like organizations of lymphocytes could be seen in cortical region (Fig. 3,4, Plate 8).

After 10 days of Con A stimulation, spaces appeared both in spleen and lymph nodes (Fig. 1,2, Plate 7; Fig. 5,6, Plate 8), possibly due to cell loss. In lymph nodes especially, the marginal sinus widened and medullary sinus appeared communicating the marginal sinus at the zone of hilus (Fig. 5, , Plate 8), and clumps of loosened and large lymphocytes could be seen in these spaces (Fig. 6, Plate 8). Number of cells per unit area of histological preparations also decreased with the appearance of these spaces (Fig.11). Even though there was cell loss by 10th day of each stimulation, the number lymphocytes again increased (Fig.11) and gave an impression of compactness (Fig. 3,4, Plate 7) at the peak hours of blastogenesis with each subsequent stimulation (Fig.10).

d) Production of antibody to Con A : As animals were stimulated repeatedly with Con A, tests in Ouchterloni plates were performed to detect anti-Con A antibody in the serum collected on 0, 30th and 50th day of injections of Con A. 0 day serum did not show any antigen-antibody reaction (Table 8) but the 30th day serum developed faint precipitation lines by reacting with Con A in the central well (Fig.1, Plate 10). Maximum detectable reaction was observed with 1:1 dilution of the serum and beyond the dilution of 1:20 there was no reaction (Table 8). Similar reactions were evident with 50th day serum also.

CON A ACTIVATED LYMPHOCYTE MEDIATED KILLING OF TUMOUR TARGET CELLS IN ^{51}CR RELEASE ASSAY :

Injection of 50 µg Con A/animal can generate cytotoxic cells both in spleen and lymph nodes (Table 9). Normal cells

not being activated with Con A in control sets showed a minimal level of cytotoxicity. The level of cytotoxicity with 24 hr of stimulation was pretty close to the control level in one experimental set and reasonably higher in another. But the percentage of cytotoxicity was always noticably higher with 48 hr of stimulation than this in control or with 24 hr of stimulation. Degree of cytotoxicity of spleen and lymph node cells were comparable. Usually increment in cytotoxicity was with the increase in number of effector cells upto 1:50 of target and effector ratio (Table 9).

Activated lymphocytes from T cell depleted animals could not mount any reasonable level of cytotoxicity, the indices were even lower than the control. Of course, it was observed that Con A could not induce a significant level of blastogenesis in T cell depleted mice (Table 3).

SUSCEPTIBILITY OF CON A TRANSFORMED BLASTS TO ANTI-THYMOCYTE SERUM :

To ascertain the thymic nature of blasts or effector cells, their susceptibility to anti-thymocyte serum plus complement mediated lysis was tested. Anti-thymocyte serum could effectively kill the blast cells from both spleen and lymph node even upto low antibody dilution of 1/640 (Table 10). This experiment was repeated six times; two representative experiments have been presented in the table.

CYCLOPHOSPHAMIDE TREATMENT AND DEPLETION OF SUPPRESSOR T CELLS FROM LYMPHOCYTE POPULATION RESPONSIVE TOWARDS TUMOUR :

To establish the effective dose of cyclophosphamide capable of removing the suppressor T cells from the lymphoid organs, four different doses like 12.5, 25, 50 and 100 mg of Cy per kg body weight per animal were injected intraperitonially and after 48 hr of injection, size and weight of spleen, mesenteric lymph node and other lymph nodes were noted. Then the increment in size and weight of secondary lymphoid organs and the percentage of blasts in these organs following the stimulation of Con A was observed. Degree of cytotoxicity of the lymphoid cells obtained from the mice treated with different doses of cyclophosphamide and stimulated with Con A was also studied in ^{51}Cr release assay.

With the treatment of cyclophosphamide there is a gradation in reduction of weight of secondary lymphoid organs (Table 11); maximum with highest dose. This effect of cyclophosphamide can easily be visualized from the size of the spleen and mesenteric lymph nodes of the treated animals (Fig.1, Plate 11 and Fig.1, Plate 12).

When the cyclophosphamide treated animals were subjected to subsequent treatment with Con A, there was increment in size and weight of the secondary lymphoid organs as shown in Fig.2 Plate 11; Fig.2, Plate 12 and Table 11. The most in-

crement was observed in case of animals treated with 25 mg of Cy/Kg. Notable increment but at lower degree were also seen in animals pretreated with 12.5 mg of Cy/Kg of body wt/animal.

Con A-induced blasts were more in number when the animals were pretreated with 25 and 12.5 mg of Cy than with two other doses of cyclophosphamide (Fig.12). The number of blasts when pretreated with 25 mg of Cy was even more than that of the normal animals. This was true with both *in vivo* and *in vitro* stimulation Con A (Fig.12a and b). Incorporation of ^{3}H-TdR by activated lymphocytes from these animals parallels the blastogenic response (Table 12).

Cytotoxicity test : As pretreatment with certain doses of cyclophosphamide causes higher level of blastogenesis (Fig.12a, b) and DNA synthesis (Table 12) in the lymphocyte population stimulated with Con A, the cytotoxic function of these cells was also tested in ^{51}Cr release assay.

The level of cytotoxicity was reasonably increased on pretreatment with two doses cyclophosphamide, 25 and 12.5 mg/kg over the normal activated cells (Table 13); the former dose was most effective. However, the degree of cytotoxicity did not increase always with the increment of the ratio of effector cells to the target cells. Cytotoxic ability of lymphocytes from animals pretreated with 100 mg Cy/kg has not been studied as Con A-induced blastogenic response of these cells was poor (Fig.12).

ADOPTIVE TRANSFER OF ACTIVATED CELLS, DEPLETED OF SUPPRESSOR T CELLS BY CYCLOPHOSPHAMIDE PRE-TREATMENT :

The aforesaid experiments indicate that cyclophosphamide pretreatment causes higher degree of blastogenesis and cytotoxicity by the residual cells. The efficacy of these cells towards the control of tumour growth was tested by transferring them at the site of tumours in syngeneic hosts at every 10 days interval. Notable suppression of tumour growth *in situ* by these cells was observed (Fig.13a). Higher number of cells in the inoculum was more effective in suppression of tumour growth. This can easily be revealed from the slopes of the lines for tumour growth in figure 13. The slower growth rate of tumour with higher dose of cell inoculum was possibly the basis of increased survivality of the host animals; 40% of the tumour bearing mice injected with 2×10^6 cells/animal, survived beyond 200 days (Fig.13b).

DISCUSSION

Immune response to malignant tumours has long been the subject of study of immunologists which received its major impetus only during 1960s. The diverse array of tumour cell surface antigens, especially on the chemically-induced tumours, always posed problem in developing specific immunological measure against malignancy. Non-specific approaches were directed mostly towards stimulating the reticulo-endothelial system of hosts with the agents containing killed microrganisms (Halpern et al, 1966; Old et al, 1969; Mathé et al, 1969b; Mitchison, 1970; Mathé, 1971; Mathe et al, 1972; Hersh, 1973; Milar et al, 1974; Purnell et al, 1976; Klein and Halterman, 1977; Hawrylko, 1977; Prim and Baldwin, 1977; Woodruff and Warner, 1977; Gupta et al, 1978; Ray et al, 1979; Fredman, 1980; Ray et al, 1980; Elkappany et al, 1980; Meyata et al, 1983) or with the help of agents of plant origin (Wall and Wani, 1977; Suffness and Douros, 1979; Fletcher et al, 1980; Jika et al, 1983). The function of graft rejection, including tumour grafts has been ascribed to the T lymphocytes (Daynes et al, 1979; Herberman et al, 1980; Keder and Weiss, 1981; Green, 1981; Prowse et al, 1983); thus stimulation of these cells possibly promises more of a specific approach. Concanavalin A (Con A) is known to activate only the T lymphocytes in such a way so that different clones of T lymphocytes can mount immune response polyspecifically. Several authors

have shown that Con A stimulates T cells *in vitro* to differentiate into polyspecific killer cells against allogeneic and tumour target cells (Stobo and Paul, 1973; Waterfield *et al*, 1975; Heinenger, 1976; Chakravarty and Clark, 1977; Green *et al*, 1978; Chakravarty, 1980). Earlier works in our and other laboratories indicated the similar phenomena even with *in vivo* stimulation of T lymphocytes by Con A (Anaclerio *et al*, 1974; Waterfield and Waterfield, 1976; Choudhuri and Chakravarty, 1981; Chakravarty and Choudhuri, 1983). Objective of our present study was to see whether such *in vivo* polyclonally stimulated lymphocytes could act against chemically-induced tumour cells, where multiplicity of cell surface antigens is encountered.

Earlier works of Choudhuri (1983) indicated that 50µg of Con A/animal was effective in activating T cells and further differentiating them into cytotoxic killer cells against allogeneic targets. Here, we have shown that 50µg of Con A/animal indeed is the optimum dose for inducing blastogenesis (Fig.2C) and characteristic level of incorporation of radioactive thymidine by the activated lymphocytes (Fig.3). Higher doses of Con A than 50µg/animal could initially boost the level of blastogenesis as it has been observed by us (Fig.2d) and Waterfield *et al*(1975) and Waterfield and Waterfield (1976), but most of these cells became physiologically exhausted and died earlier as observed by Choudhuri and Chakravarty (1981).

Gunther et al (1973) also observed nearly cent per cent death of activated lymphocytes by 72 hr after injection of more than 100 µg of Con A/animal. Thus the dose of 50 µg of Con A/animal was considered to be optimum for our purpose. The lymphocytes activated with this dose of Con A were used throughout the study to test the efficacy of polyspecifically activated lymphocytes towards MCA-induced tumour cells.

Lymphocytes activated in vivo ~~in~~ with Con A could restrict the growth of a tumour piece transplanted together in the anterior eye chamber (Group B, Table 4). The inhibition of tumour growth could easily be ascertained by the inhibition in the degree of blood vascular reactions observed upto 25 days (Plate 5), since growth of neoplastic tissue transplants is always accompanied by the increased vascularization towards the grafts (Gimbrone and Gullino, 1976; Brem et al, 1978; Ziche and Gullino, 1982). The control experiment (Group C, Table 4) showed that nonstimulated lymphocytes were not capable of inhibiting the blood vascular reactions and tumour growth significantly. It is likely that polyclonally stimulated cells caused some physiological impairement of the tumour cells in the explants so that the cells were not in a state to secrete the inducing factor (TAF) for blood vascular ramifications over cornea.

Different explanations can be put forward to account for such an inhibition of tumour growth by Con A activated

cells. It is possible that, some kind of substance(s) have been secreted by the activated lymphocytes which neutralized the tumour angiogenesis factor (TAF). Such neutralizing substances have been shown by Folkman and Cotran (1976) to be secreted from neonatal cartilage placed in between a tumour graft and blood vessels in the rabbit cornea. Lee and Langer (1983) also isolated neutralizing substance for tumour angiogenesis factor from shark cartilage. In the present investigation, the presence of activated lymphocytes in the anterior eye chamber did not only inhibit the angiogenic reactions, but also the growth of the tumour graft itself. Thus, another possibility of inhibition of angiogenesis could be the incapaciation of the tumour cells by the activated lymphocytes and in consequence tumour cells fail to secrete TAF. This possibility was initially tested by incubating the tumour pieces in vitro with Con A activated lymphocytes for 24 or 48 hr prior to their implantation in the anterior eye chamber. Such tumour piece alone was found to be incapable of inducing any significant degree of angiogenesis and growth (Group A, Table 6). Furthermore, negligible amount of incorporation of ^{3}H-TdR by the tumour pieces after incubation with activated lymphocytes in vitro indicated the possible incapaciation of tumour cells (Fig.4). To test whether this physiological incapaciation was due to cell death, we tested the cytotoxicity of Con A activated lymphocytes against the ^{51}Cr labelled tumour target cells, the results will be discussed

later.

Con A stimulated lymphocytes restricted the tumour growth not only in the anterior eye chamber but also *in situ* on adoptive transfer at tumour site. Highest cell dose, 2×10^6 cell/animal was most effective in inhibiting the growth of the tumour at its site and in consequence increased the life span of the hosts (Fig.7a,b). Passive transfer of lymphocytes, specially immuned to combat the growth of respective solid tumours without much success (Delorme and Alexander, 1964; Burton and Warner, 1977; Broberg *et al*, 1972; Rossenberg and Terry, 1977; Fernandez and Feldman, 1980; Prowse *et al*, 1983). So far there was not many reports about using nonspecifically raised lymphocytes for adoptive transfer; however, some success was achieved by transfer of T cells expanded in T cells growth factor or in interleukin 2 (Eberlein *et al*, 1982; Eberlein *et al*, 1982; Mazumder and Rosenberg, 1984). Possibly our approach of raising effector cells by *in vivo* use of a polyclonal T cell stimulator cut much short the methodical paraphernalias in adoptive transfer to combat the tumour growth with some degree of success. However, the number of times of cell transfer can possibly be reduced than used in the present study. Further study needs to be initiated in this direction.

Then it was obvious to try to stimulate the lymphocytes of tumour bearing hosts as a more direct approach. Host's immune system was stimulated following two protocols. First, Con A was injected only twice, 5 days prior and 5 days later to MCA injection; this was not significantly effective in

curbing the tumour growth (Fig.8). The second protocol was to stimulate repeatedly the host's immune systemed by injecting Con A at every 10 days for 12 times beginning from the palpable appearance of tumour. The second protocol (Fig.9a,b) was found to be comparatively better than the first one (Fig.9), but this schedule also opens the possibility of clonal exhaustion and generation of antibody to Con A due to repeated injection of it.

The possibilities were tested by repeated injections of Con A for 5 times in normal non-tumour bearing animals following the same schedule as was in second protocol. Similar percentage of blast cells at 48 hr after each of the five stimulations with Con A (Fig.10) indicated the presence of restimulatable lymphocytes bothiin spleen and lymph node and thus speaks against clonal exhaustion of responder cells due to repeated stimulations, at least for five times (Fig.1,2, Plate 9). However, very low titre of anti-Con A antibody could be detected only upto 1/20th dilution of the serum from animals after three injections of Con A (Table 8). Besides these, for the possible adverse effect of repeated injections of Con A, the histological study of lymphoid organs and growth of the animals in term of body weight were taken into consideration. The blastoid transformation of the lymphocytes throughout the spleens and lymph node with each stimulation were easily discernible from the histological preparations. In between the

stimulations, number of lymphocytes in the secondary lymphoid organs were comparable per area basis to that of the normal mouse (Fig.11) and the total cell count of spleen or lymph node did not alter significantly (Table 7). Cell loss and appearance of space in the secondary lymphoid organs (Fig.1, 2, Plate 7 and Fig.5,6, Plate 8) following the peaks of blastogenesis was observed and these were possibly an indicative of cell flow out from the organs as a possible natural phenomenon following higher level of blastogenesis. Furthermore, the body weight of the animals in course of repeated stimulation did not reflect any adverse effect of the treatment (Table 7).

However, our observations indicated that repeated transfer of Con A activated syngeneic lymphocytes at the tumour site (Fig. 7) was more effective than the repeated stimulation of host's own system with Con A (Fig. 8,9). Out of several possibilities this could be due to the low titre of antibody to Con A (Table 8, Fig.1, Plate 10) generated in the hosts which neutralized a part of the Con A injected every time. It may also be possible that the injected Con A bind to its receptors on the tumour cells and produce a masking effect so that the tumour cells may escape the destruction by the activated lymphocytes of host origin. Although, repeated <u>in vivo</u> injections of Con A in the tumour bearing mice could not very significantly curb the growth rate of tumour as it was observed with repeated injections of activated syngeneic lymphocytes, at least this schedule could obviously make some of the T lympho-

cytes in the hosts reactive to the malignant cells, which were otherwise non-responsive, possibly due to some suppressor factor(s) or blocking factor of host origin. There is the possibility of generation of inhibiting factor for effector lymphocytes by the malignant cells which could lessen the degfee of function of the activated lymphocytes of the tumour bearing mice or the adoptively transferred T lymphocytes. Several authors have already shown the existence of such suppressor factors (Hellström and Hellström, 1970; Baldwin et al, 1972; Gershon et al, 1972; Happner et al, 1973; Hellström et al, 1973; Gershon et al, 1974; Herberman, 1974; Baldwin et al, 1975; Fujimoto et al, 1976; Bansal, 1976; Hellström and Hellström, 1978b; Hellström and Hellström, 1979; Naor, 1980; Ray and Saha, 1982). The aspect of generation of inhibiting factor(s) by malignant cells was not taken into much consideration in the present investigation to avoid further complications in working out the set objective of this study. But this will be dealt in future in some model in vitro experiments, like assaying the blastogenic and cytotoxic responses of the lymphocytes from normal and tumour bearing mice in presence of serum of tumour bearing host and factors produced by tumour explants in culture.

Mechanisms involved in restriction of tumour growth by the Con A activated lymphocytes both in the anterior eye chamber and in situ, could possibly be explained by the cytotoxic nature of the activated T cells as revealed in the

^{51}Cr release assay. We observed that radioactivity released in the supernatant was higher when Con A activated lymphocytes were added to the labelled tumour target cells (Table 9) in comparison to the control where normal non-activated lymphocytes were added to the assay tubes. The higher level of chromium release is usually an indicative of death of a greater number of target cell. Earlier observations indicated that both *in vitro* (Clark, 1975; Bevan and Cohn, 1975; Heinenger *et al*, 1976; Chakravarty and Clark, 1977; Green *et al*, 1978; Chakravarty, 1980) and *in vivo* (Waterfield *et al*, 1975; Anaclario, 1974; Choudhuri, 1983) activation with Con A leads to the cytotoxic differention of lymphocytes. Significant level of cytotoxicity was thus revealed by the activated cells after 48 hr than 24 hr of injection of Con A. Choudhuri and Chakravarty (1983) also observed highest level of cytotoxicity at 48 hr of Con A activation of the lymphocytes from C57BL/6 mice against allogeneic target cells of DBA/2 origin. By this time of cytotoxic differentiation an appreciable percentage of lymphocytes expectedly transformed into blasts and could incorporate a significant level of ^{3}H-TdR (Fig. 2C and Fig. 3).

The cytotoxic response was possibly mediated by T cells as revealed from the fact that removal of T cells before activation with Con A abrogated the cytotoxic response of the residual cells (Table 9). Indeed, the number of blast cells in the residual population of cells treated with Con A was also very poor

(Table 3). The T cell nature of the Con A responsive cells was further confirmed from the fact that the Con A transformed blast cells are susceptible to lysis by anti-thymocyte serum (Table 10). T cell depleted lymph node piece treated with Con A also failed to inhibit the tumour induced angiogenesis and tumour growth in the anterior eye chamber (Group B Table 5) indicating more so the participation of cytotoxic T cells in the inhibition of tumour growth.

Of course, the question about the participation of natural killer (NK) cells remains. It is known that, NK cells in the normal lymphoid population can mount spontaneous in vitro cytotoxic reaction against variety of tumour target cells without prior immunization (Herberman et al, 1975a; Kiessling et al, 1975; Takasugi et al, 1973; Herberman et al, 1978; Karre et al, 1980; Gershon et al, 1981). Although these NK cells possess Fc-receptors for IgG (Herberman et al, 1977; Pape et al, 1977; West et al, 1977) and a low density of Thy-1 antigen on their surface (Herberman et al, 1975b; Herberman, 1978; Herberman et al, 1978), Dennert (1980) and Zarling (1980) could clearly distinguish them from the killer T cells with the help of monoclonal antibodies to Lyt antigens in mouse and OKT3, OKT8 and OKM1 antigens in man. In our cytotoxic assays, the possibility remains that the background level of cytotoxicity with non-activated lymphocytes may be due to NK cells, the level of which was significantly lower than that observed with activated cells at 48 hr (Table 9). However, further experi-

ments need to be performed to know whether Con A treatment can make NK cells more effective in their response.

A polyclonal stimulator of T cells like Con A is supposed to activate all the subsets of T lymphocytes including the suppressor one. Workers like Gershon et al (1972), Gershon (1974), Cantor and Simpson (1975), Tada (1975), and Hodes and Hatchock (1976) have shown that these suppressor cells are responsible to modulate immune responses in normal animals. This subset of T cells are also known facilitate the growth of tumour by interacting with the immune responsive cells in a tumour bearing host (Gershon et al, 1974; Fujimoto et al, 1975; Fujimoto et al, 1976; Border et al, 1977; Hellström and Hellström, 1978; Naor, 1980; Green, 1980; North and Bursuker, 1984). Use of higher doses of Con A like 25 mg or more per ml in vitro have been implicated by Peavy and Pierce (1974), Shou et al (1976), Primi et al (1979) and Ozer et al, (1982) for enhancing suppressor activity of T cells in cytotoxic responses. In vivo, the dose of 150 µg Con A or more injected per animal has been shown to enhance the activity of the suppressor cells both in normal and tumour bearing mice (Nirmul et al, 1972; Markowitz et al, 1969; Dutton, 1973; Egans et al, al, 1974; Davis et al, 1975; Nespolie et al, 1977; Primi et al, 1979; Ekstedt, 1979; Eibl et al, 1980; Catalona et al, 1980). Although, one third of this amount of Con A was injected in vivo in the present study, the possibility of activating the suppressor T cells remained. So, in certain experimental sets,

suppressor T cells were removed by cyclophosphamide treatment prior to activation and then degree of function of this residual activated cells were tested in cytotoxicity and adoptive transfer assays.

Test with different doses of cyclophosphamide was necessary as the dose and the schedule of administration was usually considered to be critical in effective removal of suppressor T cells. Workers like Winkelstein (1973), Dumont (1974), Putman et al, (1975), McIntosh et al (1979), Greely et al (1982), Wilmer et al, (1984) and Greely et al (1985) observed that a dose more than 100 mg of cyclophosphamide/kg body weight/animal often produced a general immune suppression and toxic effect, on the other hand lower doses of cyclophosphamide were shown to remove selectively the suppressor subpopulation of T lymphocytes without causing much toxicity to other cells (Gvaser, 1979; Kauffman et al, 1980; Stevenson and Fauci, 1980; Ray and Roychoudhuri, 1981; Yu et al, 1980; Berd et al, 1984; Ye and Mokyr, 1984). Again the treatment with cyclophosphamide prior to antigenic challenge was found to be more effective than a post antigenic treatment (Bonavida, 1977; Yu et al, 1980; North, 1982) and hence the schedule of cyclophosphamide treatment 48 hr prior to Con A injection was chosen here. Out of the four doses of cyclophosphamide (100, 50, 25, and 12.5 mg/kg body wt/animal) tried in this study, the dose of 25 mg/kg was considered to be most effective. This was found on the basis of higher percent of blast cells

(Fig.12) with higher degree of cytotoxicity (Table 13). Whereas the doses higher than that reduced the size and weight of the lymphoid organs (Table 11) to such an extent that could not be brought even to the normal level after Con A treatment, possibly due to low blastogenic transformations (Fig. 12).

Thus it seems that even the dose of 50 µg of Con A/ animal could generate suppressor T cells and effective removal of these cells prior to Con A stimulation indeed made the effector cell population more efficient in cytotoxic killing (Table 13). Possibly, reflection of this effective killing by Con A activated cells in the absence of suppressor T cells was observed in the restriction of tumour growth and increasing the life span of hosts on adoptive transfer of these cells at the tumour site (Fig. 13 a,b).

In conclusion, this study provides some evidences that polyclonal stimulation of T cells with Con A can generate the effector cells to combat chemically-induced tumours in mice. This is possibly a hopeful proposition in the backdrop of failures to develop any specific immunity against neoplastic cells with diverse types of tumour associated antigens. Inhibition in angiogenesis and tumour growth in the anterior eye chamber or <u>in situ</u> in presence of Con A activated cells was possibly the reflection of killing of tumour cells by poly-

clonally stimulated lymphocytes as evident from ^{51}Cr release cytotoxic assay. The adoptive transfer of activated cells at the site of the tumours was more effective over *in situ* stimulation of host's lymphocytes; this could be due to several factors including low titre of antibody towards Con A.

This study suggests the feasibility of making this model of polyclonal stimulation of lymphocytes operative in human.

SUMMARY

As because the tumour cells possess diverse types of antigens (TAAs) on their surface, the possibility of immune response of polyclonally stimulated lymphocytes towards the tumour target cells was studied in the present investigation. First, the efficacy of polyclonally stimulated cells was tested by co-grafting them with a tumour piece explant in the anterior eye chamber of a syngeneic animal where a tumour piece transplanted alone can induce neovascular reactions or angiogenesis. Then, the stimulated cells were injected at the tumour site to note their ability in curbing the growth of the tumour. In another experimental series, the lymphocytes of the tumour bearing hosts were directly stimulated. Cytotoxic ability of the polyclonally stimulated lymphocytes towards tumour target cells was observed in ^{51}Cr release assay. As, a polyclonal T cell stimulator is supposed to activate the suppressor subset of T cells also, in one set of experiments activation of T lymphocytes was done *in vivo* after removal of suppressor T cells with cyclophosphamide treatment and their response towards tumour cells were tested in *in vitro* cytotoxic assays and in *in vivo* adoptive transfer experiments.

Following the earlier studies, we found that the dose of 50 µg of the plant lectin Concanavalin A (Con A) injected in a mouse is optimal to stimulate the T cells in terms of blastogenesis and DNA synthesis. The tumours were induced by

using 2 mg of 3'-Methylcholanthrene per animal subcutaneously. After several trials with different doses and different makes of MCA, the dose of 2 mg of MCA (Sigma Chem. Co., St. Louis, USA) per animal was found most effective in inducing fibrosarcimas in terms of early appearance and faster rate of growth of tumours.

A tumour piece transplanted in the anterior eye chamber of mouse can induce neo-vascular reactions like vasodilatation and appearance of new vessels and their branches in two phases over the cornea. A piece of lymph node containing Con A activated lymphocytes, grafted along with a tumour piece can dramatically inhibit these reactions and growth of tumour observed upto 25 days. Non-stimulated lymphocytes in control experiments could not inhibit these reactions induced by a tumour explant.(Plate 5).

The possibility of incapaciation of tumour cells by the Con A activated cells was tested by incubating the tumour explants with these effector cells in vitro for 24 and 48 hr. Incubation with Con A activated lymphocytes reduced significantly the ability of tumour pieces to induce angiogenic reactions over the cornea and to grow in the anterior chamber of eye. Their ability to incorporate radioactivity in vitro was also curbed to a great extent after incubation with stimulated lymphocytes.

The efficacy of these polyclonally stimulated cells to curb tumour growth was also studied after transferring them at

the tumour site and then measuring the size of tumours and the life span of the hosts. Three different concentrations of cells (5×10^5, 10^6 and 2×10^6/animal), was tried, of which the dose of 2×10^6 cells/animal appeared to be most effective in retarding the tumour growth; this is obvious from the slopes of the lines drawn for tumour growth. The slower growth rate of tumour enabled 15% hosts to survive beyond 150 days whereas all the control animals died within 98 days.

These observations led to an approach of stimulating hosts own lymphocytes to combat the malignant growth. Two i.v. injections of Con A, one 5 days prior to and another after 5 days of tumour induction with MCA was made, which could not influence the tumour growth very significantly. Whereas, repeated Con A injections at 10 days intervals, for 12 times starting from the day of palpable appearance of tumour inhibited the growth rate of tumour to a great extent and thereby increased the life span of the hosts.

The possibility of clonal exhaustion of responsive lymphocytes due to such repeated stimulation with a polyclonal stimulator was analysed by using normal mice and noting the changes in cellular organization of their lymphoid organs. Percentages of blasts with repeated stimulations were comparable to that of normal mice stimulated once. At the peak hour of blastogenesis the lymphocytes in spleen and lymph nodes increased in number and after that, some of them were found in the

lacunae or spaces of the lymphoid organs in histological preparations as if they were flowing out of the lymphoid organs. However, the total lymphocyte content in these organs and in per ml of peripheral blood after the wane of each stimulation, usually did not fall below the values of normal non-injected animals. Body weight of the experimental animals during the course of the injections was same as in control normal animals indicating that there was possibly not much adverse effect of repeated injections on physiology of the animals. Very low titre of antibody to Con A was detectable after three times injections of Con A and discussion has been made whether this could be one of the reasons to make repeated injections of Con A *in vivo* to stimulate host's lymphocytes comparatively less effective than adoptive transfer of effector cells directly at the tumour site.

Cytotoxic ability of these activated lymphocytes to kill radiolabelled tumour target cells in *in vitro* assay suggests the possible mechanism of immune response of Con A stimulated lymphocytes to 3'-Methylcholanthrene-induced tumour cells. Normal non-activated lymphocytes could only mount a very limited cytotoxic reaction to the tumour target cells. The possible involvement of natural killer cells in background level response has been discussed.

Removal of T cells prior to activation always abrogated the response of activated lymphocytes both in cytotoxic assays and in anterior eye chamber transplantation experiments.

Elimination of T cells was done by neonatal thymectomy and subsequent treatment of these animals with anti-mouse thymocyte serum raised in rabbit. Indeed, poor generation of blasts like 16 to 18 percent was noticeable after 48 hr of Con A injection in the animals depleted of T cells. The T cell nature of the Con A responsive cells was further confirmed by their susceptibility to the anti-thymocyte serum *in vitro*.

Although, far less the amount of Con A than the doses usually responsible for suppressor cell generation was used for lymphocytic stimulation in the present investigation, it was observed that depletion of suppressor cells with cyclophosphamide pretreatment augmented considerably the immune response of the residual T cells in cytotoxic assays and in curbing the tumour growth *in situ* on adoptive transfer. Out of four different doses, the dose of 25 mg of cyclophosphamide per kg body weight of an animal was found to be optimal for the purpose on the basis of its effectiveness to enhance the Con A response in terms of *in vivo* and *in vitro* blastogenesis. Increase in *in vivo* Con A induced blastogenesis was reflected in the increase in size and weight of the secondary lymphoid organs of the animals pretreated with cyclophosphamide and accompanied increase in ^{3}H-TdR uptake by the lymphocytes.

Feasibility of extending this model of polyclonal stimulation of T cells in other animal systems including man to combat malignancies has been suggested here.

Figure 1. Rate of growth of tumour induced by different doses of 3'-Methyl-cholanthrene of two different brands.

a) Koch-Light Laboratories Ltd., Colnbrook, U.K. and b) Sigma Chemical Co., St. Louis, U.S.A.

Dose indicated is for per animal and 'n' on the lines indicate the initial number of animals used for testing a particular dose. Size of the tumours were measured every 7 days (considering the day of MCA injection as 0 day). The Lines were drawn according to least square fit method and the slopes were calculated. Observed values for Y upto 4.0 have been plotted in the figure.

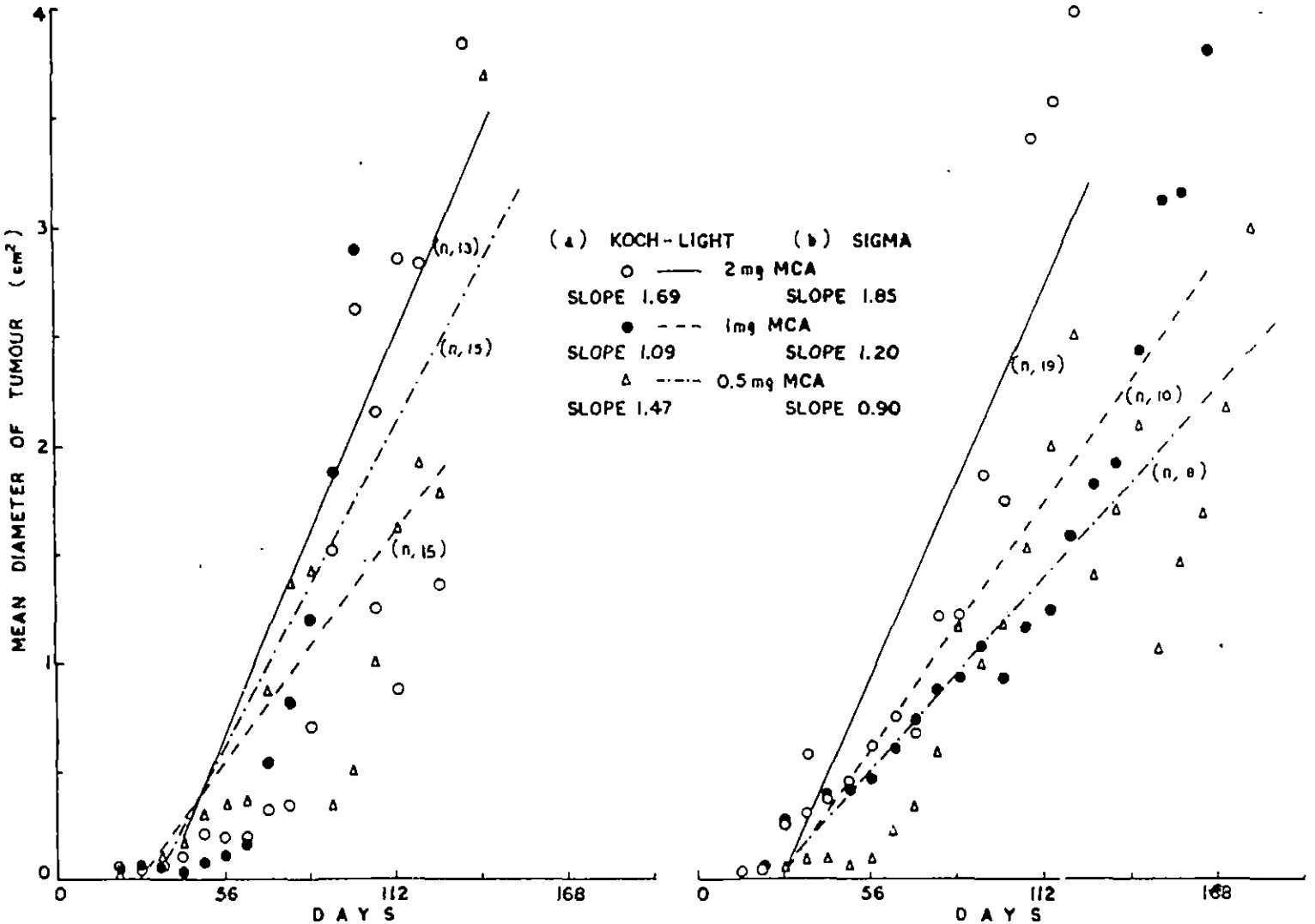

MEAN DIAMETER OF TUMOUR (cm²)
0
1
2
3
4
0
56
112
168
DAYS
(a) KOCH-LIGHT
(b) SIGMA
2 mg MCA
SLOPE 1.69
SLOPE 1.85
1mg MCA
SLOPE 1.09
SLOPE 1.20
0.5 mg MCA
SLOPE 1.47
SLOPE 0.90
(n, 13)
(n, 15)
(n, 15)
(n, 19)
(n, 10)
(n, 8)

Figure 2. Kinetics of blastogenesis of lymphocytes from secondary lymphoid organs and peripheral blood after *in vivo* treatment with different doses of Con A.

a) 10 µg and b) 20 µg per animal

●———● Spleen, ○———○ Mesenteric lymph node, o———o other lymph nodes and ▲———▲ Peripheral blood.

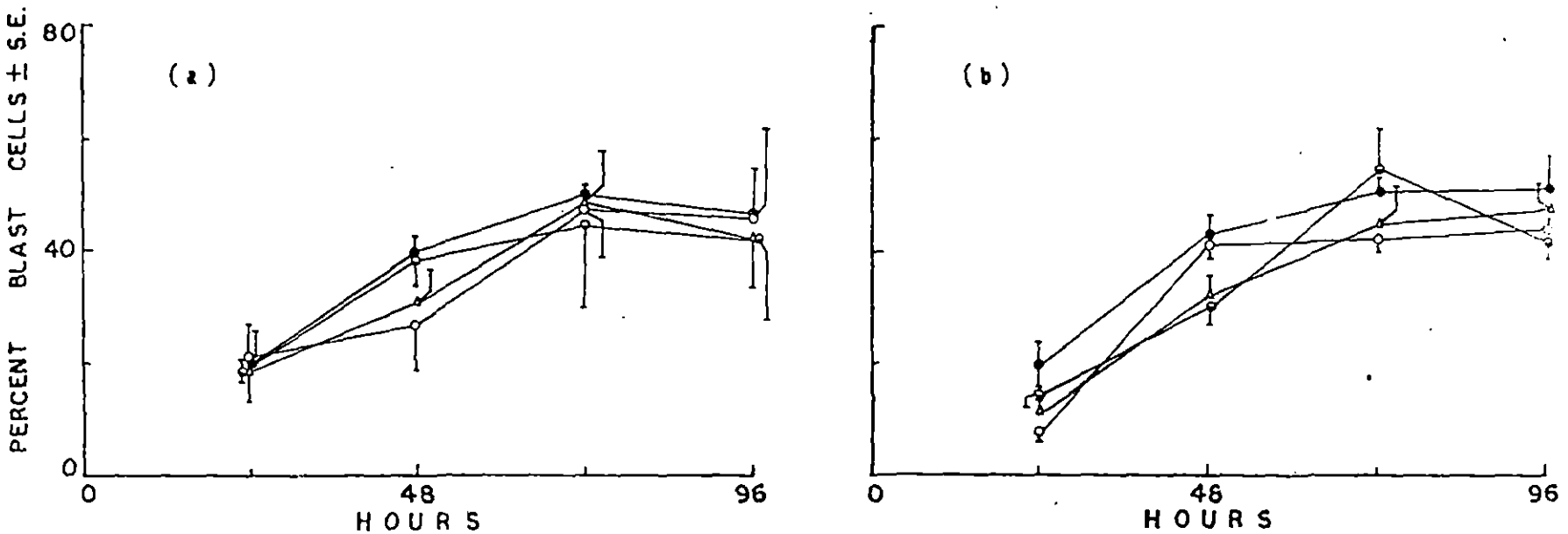

PERCENT BLAST CELLS ± S.E.
80
40
0
(a)
0
48
96
HOURS
(b)
0
48
96
HOURS

Figure 2. Kinetics of blastogenesis of lymphocytes from secondary lymphoid organs and peripheral blood after *in vivo* treatment with different doses of Con A.

c) 50 µg and d) 100 µg per animal

(●——●) Spleen, ■——■ Mesenteric lymph node, o——o other lymph nodes and ▲——▲ Peripheral blood.

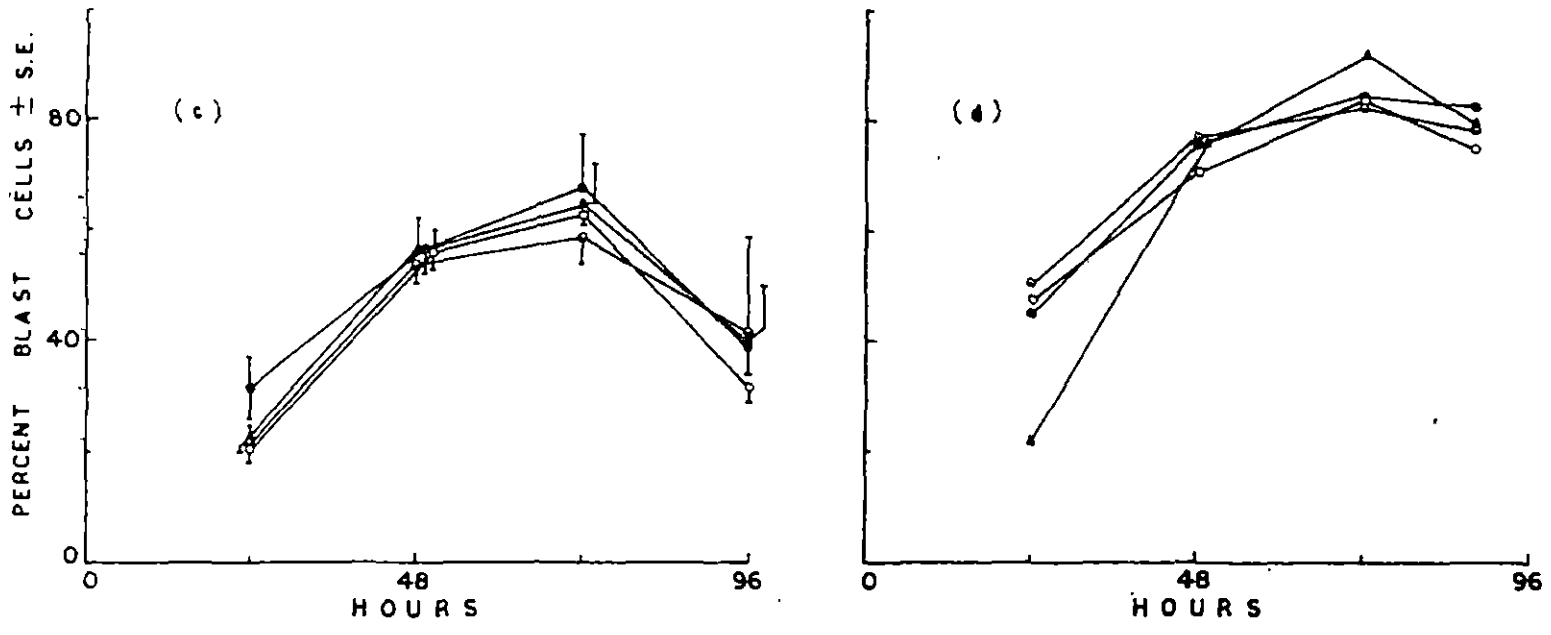

PERCENT BLAST CELLS ± S.E.
80
40
0
(c)
0
48
96
HOURS
(d)
0
48
96
HOURS

Figure 3. Incorporation of ^{3}H-TdR by the lymphocytes from spleens and mesenteric lymph nodes at different hours after *in vivo* stimulation with 50 µg of Con A/animal.

●————● Spleenocytes and o---------o Mesenteric lymph node cells.

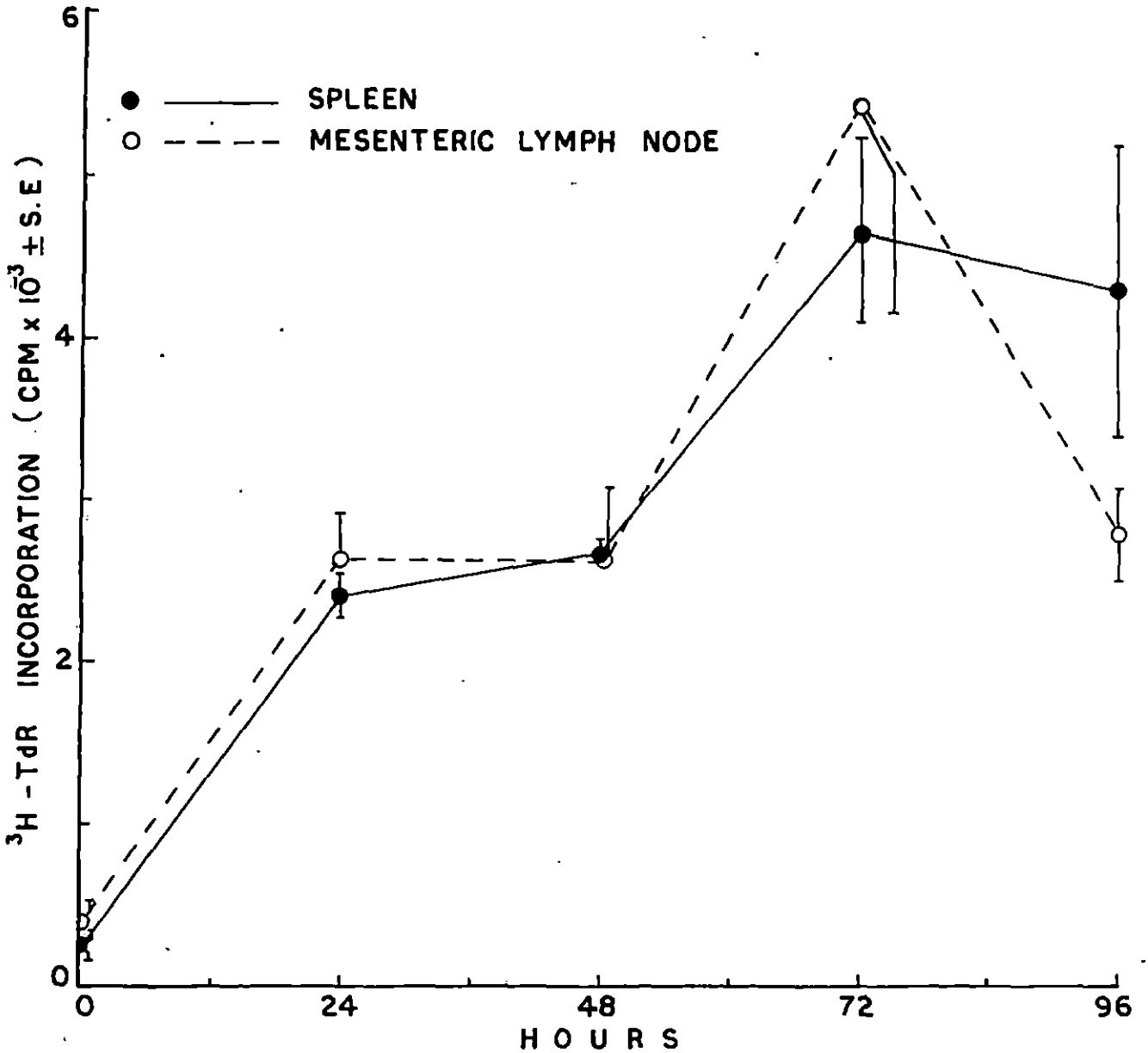

SPLEEN
MESENTERIC LYMPH NODE
^{3}H -TdR INCORPORATION (CPM x 10^{-3} ± S.E)
0
2
4
6
0
24
48
72
96
HOURS

Figure 4. Incorporation of ^{3}H-TdR by the tumour pieces after 24 or 48 hr of incubation with Con A activated or non-activated lymphocytes.

Control 1 - Fresh tumour pieces, Control 2 - Tumour pieces incubated without any lymphocytes. The level of background count was at 40 (.....).

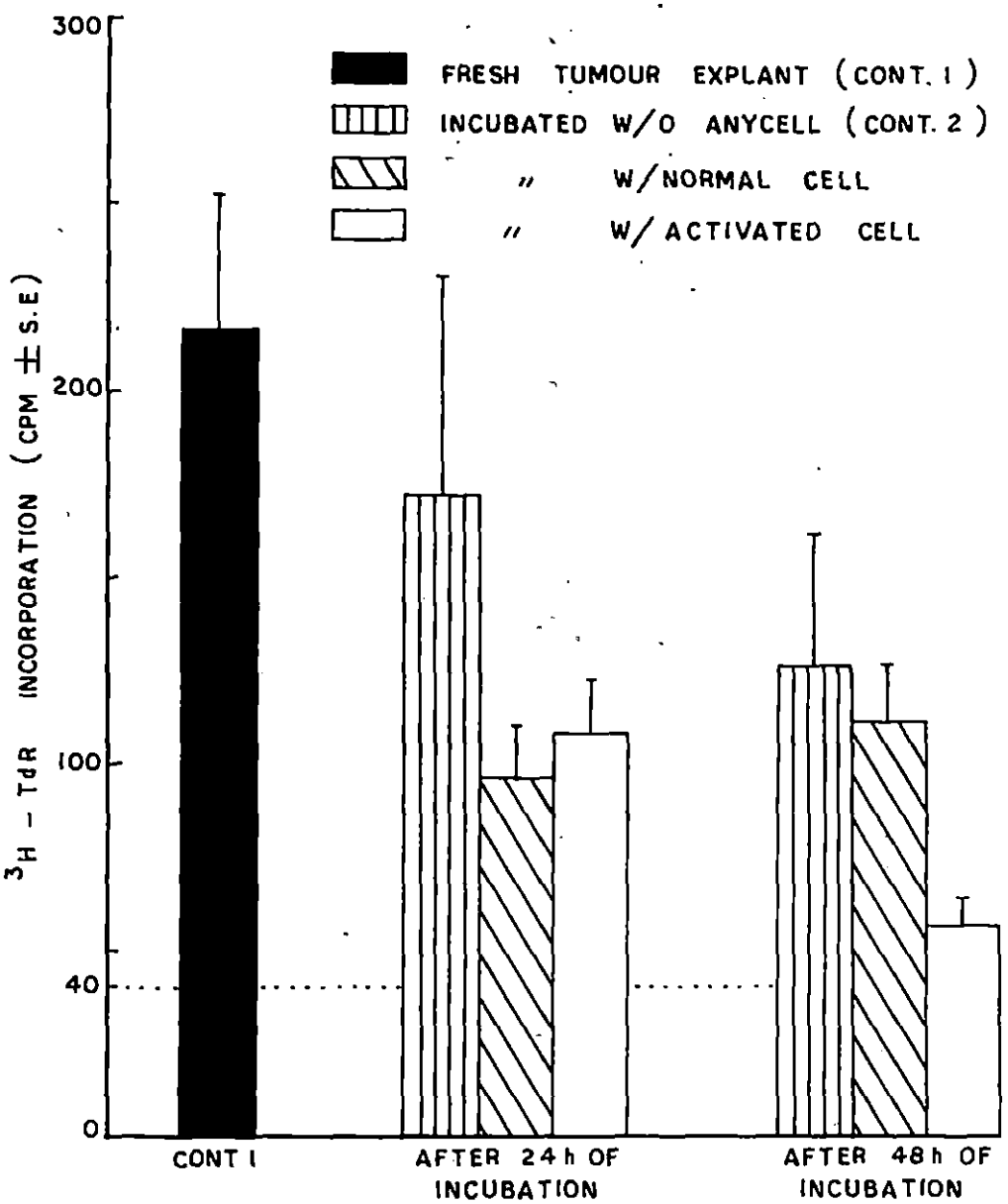
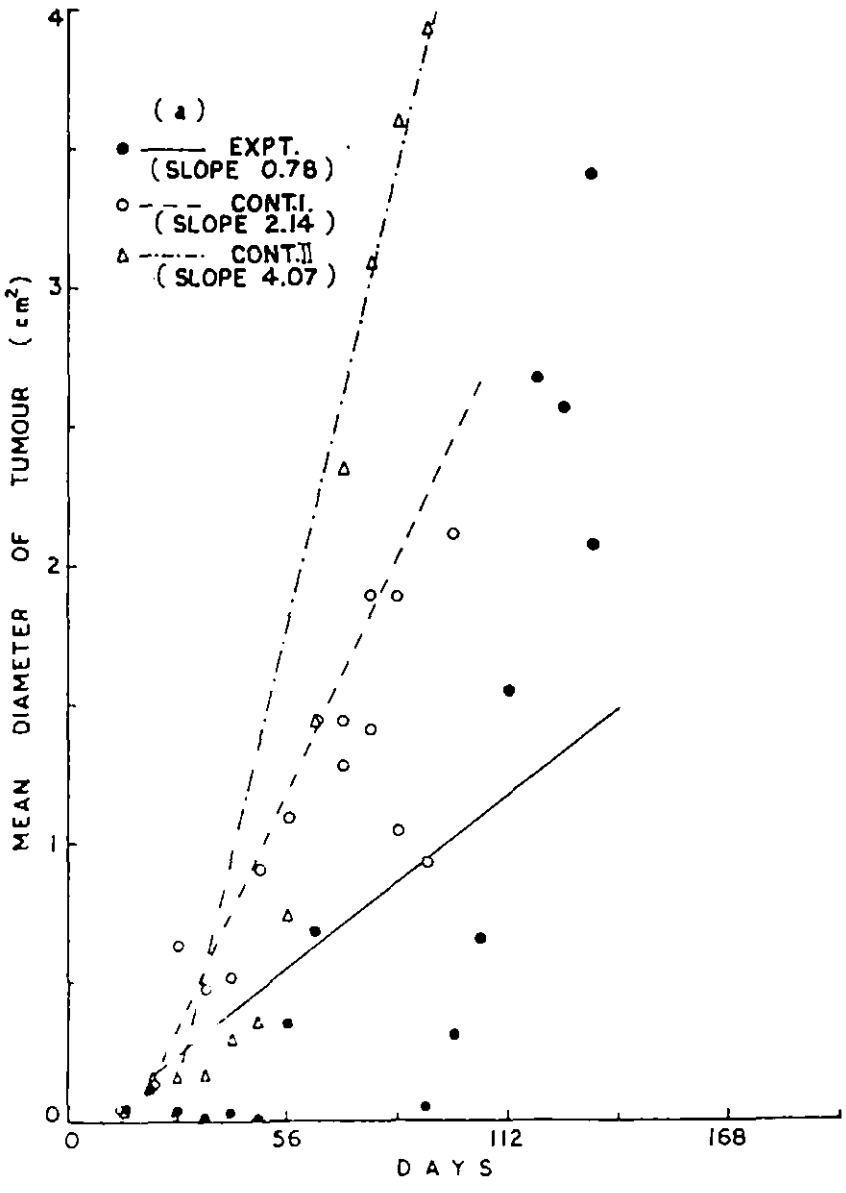

300
200
100
40
0
^3H - TdR INCORPORATION (CPM ± S.E)
FRESH TUMOUR EXPLANT (CONT. 1)
INCUBATED W/O ANYCELL (CONT. 2)
" W/NORMAL CELL
" W/ ACTIVATED CELL
CONT I
AFTER 24 h OF INCUBATION
AFTER 48 h OF INCUBATION

Figure 5a. Rate of tumour growth after injections of Con A activated lymphocytes at the tumour site, measured every 7 days (day of MCA injection considered 0 day). The lines are drawn according to least square fit method. Values for Y upto 4.0 have been plotted in the figure. Experimental (●———) animals injected with 5×10^5 activated lymphocytes every 10 days for 12 times, Control I (o-------) animals injected with normal non-activated lymphocytes following the same dose and schedule, Control II (Δ.....) without any injection.

b. Percentage of survival on different days of tumour bearing mice with 5×10^5 activated cells/animal (Expt.) and non-activated cells (Cont.) following the above ~~animals~~ schedule.


(a)
EXPT.
(SLOPE 0.78)
CONT.I.
(SLOPE 2.14)
CONT.II
(SLOPE 4.07)
MEAN DIAMETER OF TUMOUR (cm²)
0
1
2
3
4
0
56
112
168
DAYS

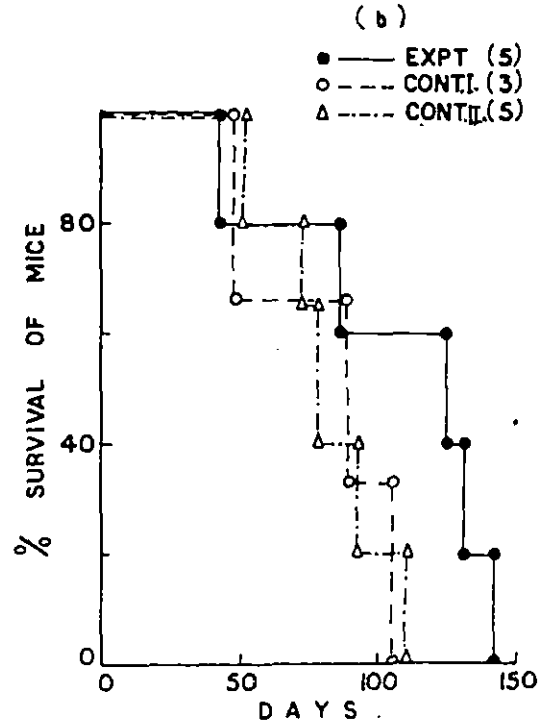

(b)
EXPT (5)
CONT.I. (3)
CONT.II. (5)
% SURVIVAL OF MICE
0
40
80
0
50
100
150
DAYS

Figure 6a. Rate of tumour growth after injections of Con A activated lymphocytes at the tumour site, measured every 7 days (day of MCA injection considered 0 day). The lines are drawn according to least square fit method.- Values for Y upto 4.0 have been plotted in the figure.

Experimental (●——————) animals were injected with 10^6 activated cells/animal every 10 days for 12 times, Control (o---------) animals injected with normal non-activated lymphocytes following the same dose and schedule.

b. Percentage of survival on different days of tumour bearing mice injected with 10^6 activated cells/animal (Expt.) and non-activated lymphocytes (Cont.) following the above schedule.

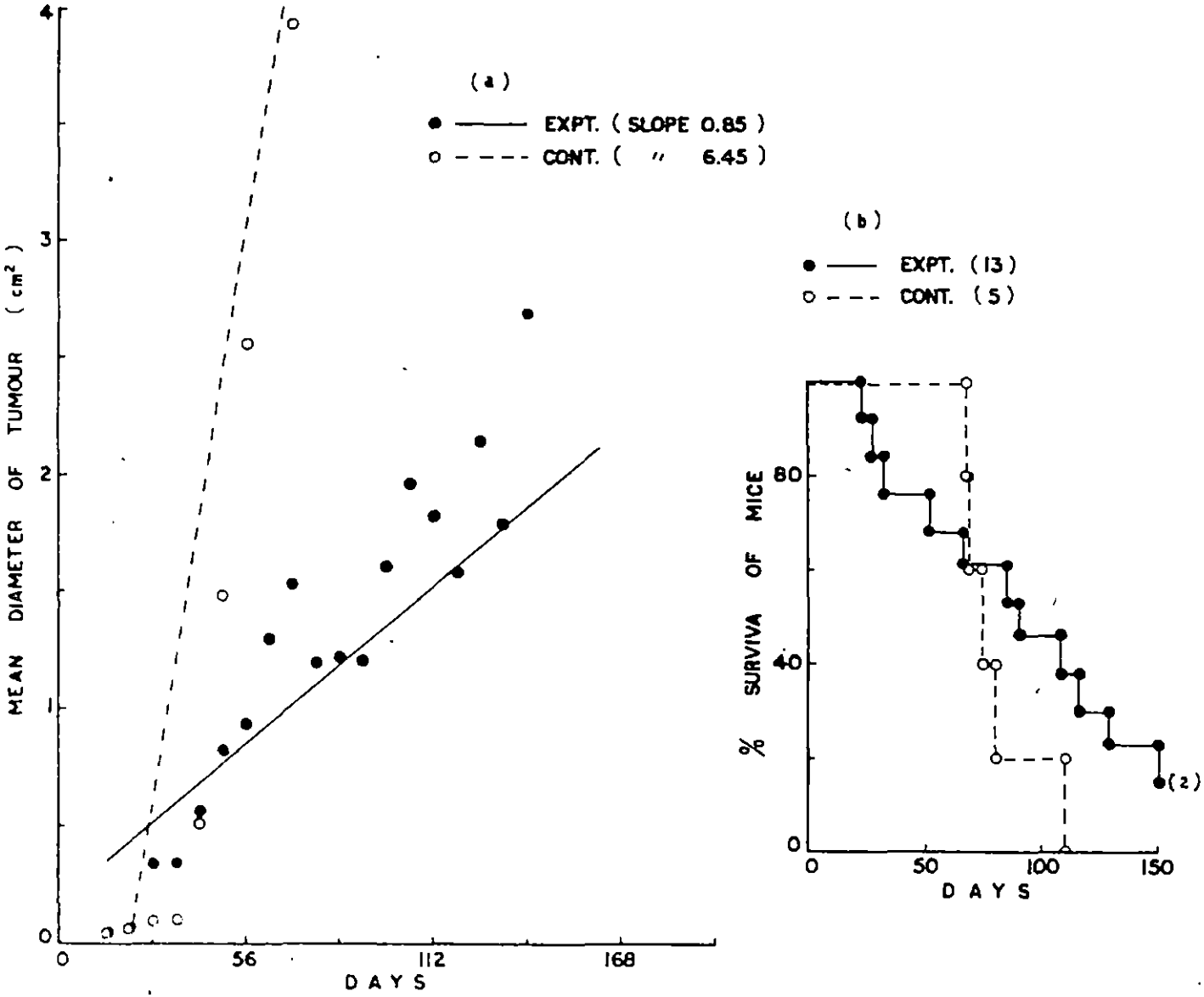
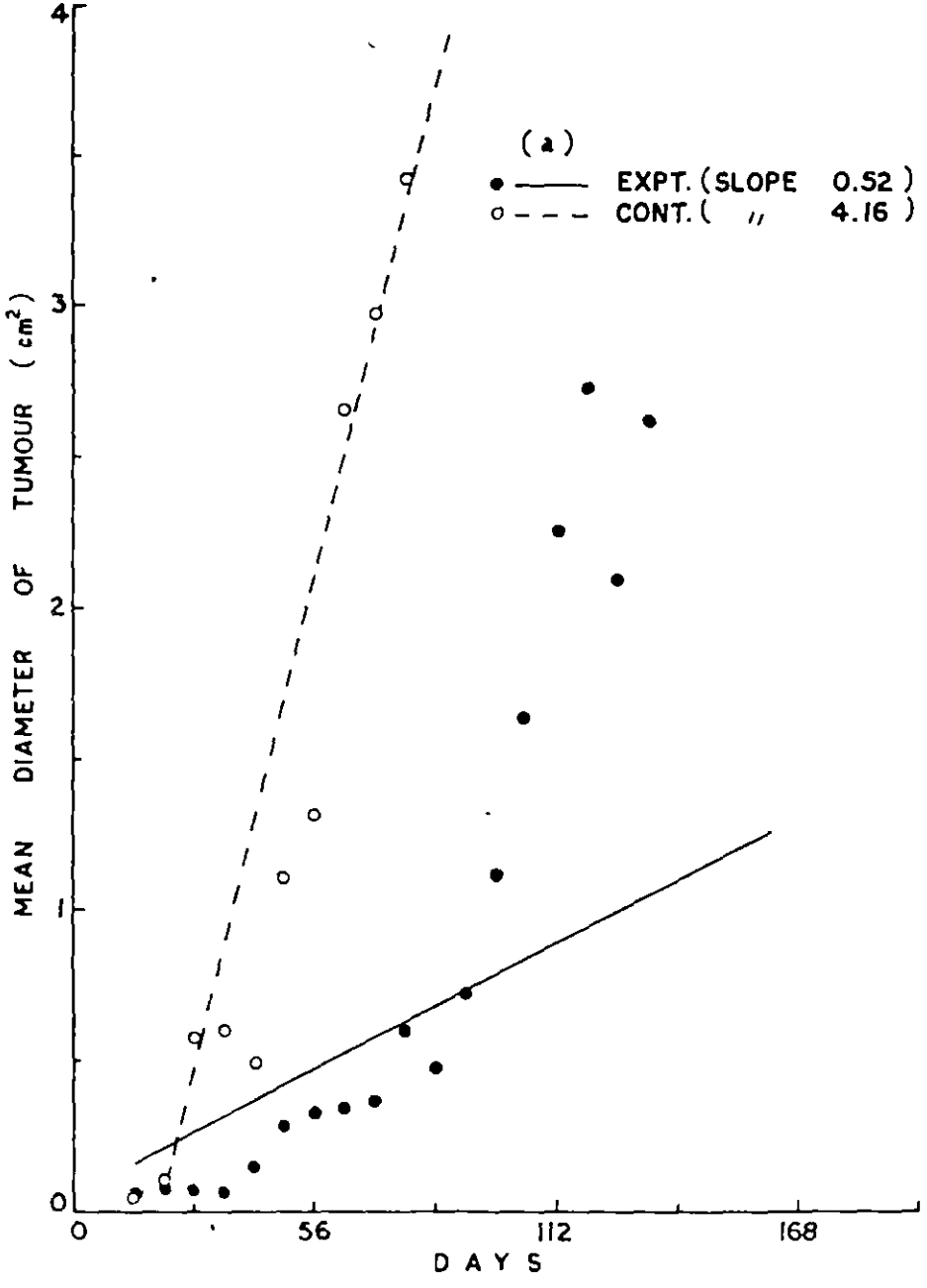

(a)
EXPT. (SLOPE 0.85)
CONT. (" 6.45)
MEAN DIAMETER OF TUMOUR (cm²)
0
1
2
3
4
0
56
112
168
DAYS
(b)
EXPT. (13)
CONT. (5)
% SURVIVA OF MICE
0
40
80
0
50
100
150
DAYS
(2)

Figure 7a. Rate of tumour growth after injection of Con A activated lymphocytes at the tumour site, measured every 7 days (day of MCA injection considered 0 day). The lines were drawn according to least square fit method and slopes were calculated. Observed values for Y upto 4.0 have been plotted in the figure.

Experimental (●————) animals were injected with 2 x 10^6 activated cells/animal and Control (o---------) animals were injected with normal non-activated cells following the same dose and schedule.

b. Percentage of survival on different days of tumour bearing mice injected with 2 x 10^6 activated cells/animal (Expt.) and non-activated lymphocytes (Cont.) following the above schedule.


(a)
EXPT. (SLOPE 0.52)
CONT. (" 4.16)
MEAN DIAMETER OF TUMOUR (cm²)
0
1
2
3
4
0
56
112
168
DAYS

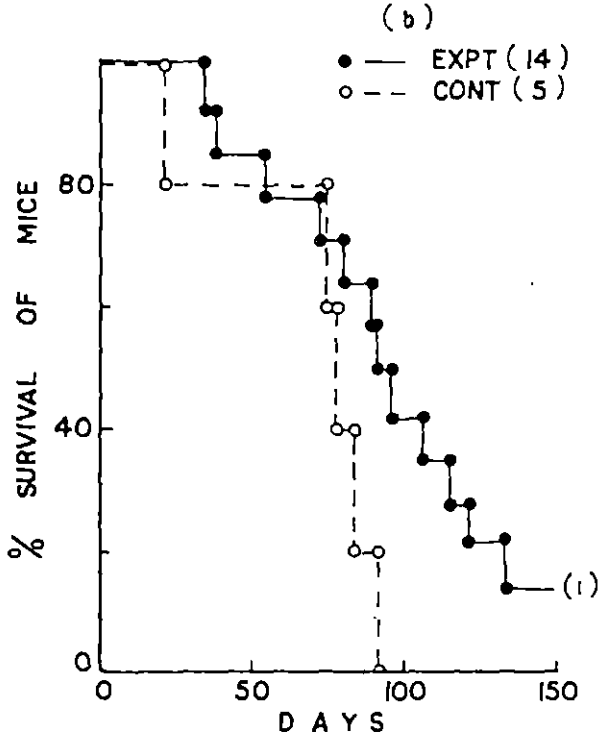

(b)
EXPT (14)
CONT (5)
% SURVIVAL OF MICE
0
40
80
0
50
100
150
DAYS
(1)

Figure 8a. Rate of tumour growth after intravenous injections of Con A, measured every 7 days (day of MCA injection considered 0 day). The lines were drawn according to least square fit method. Values for Y upto 4.0 have been plotted in the figure.

Experimental (●———) animals injected twice with Con A on -5 and +5 day of MCA injection, Control (o-------) injected with normal saline following the same schedule.

b. Percentage of survival on different days of tumour bearing mice injected with Con A (Expt.) and normal saline (Cont.) following the above schedule. Number in the parentheses indicates the number of animals at the beginning and the number at the end of the plotting indicates the number of animals at that point.

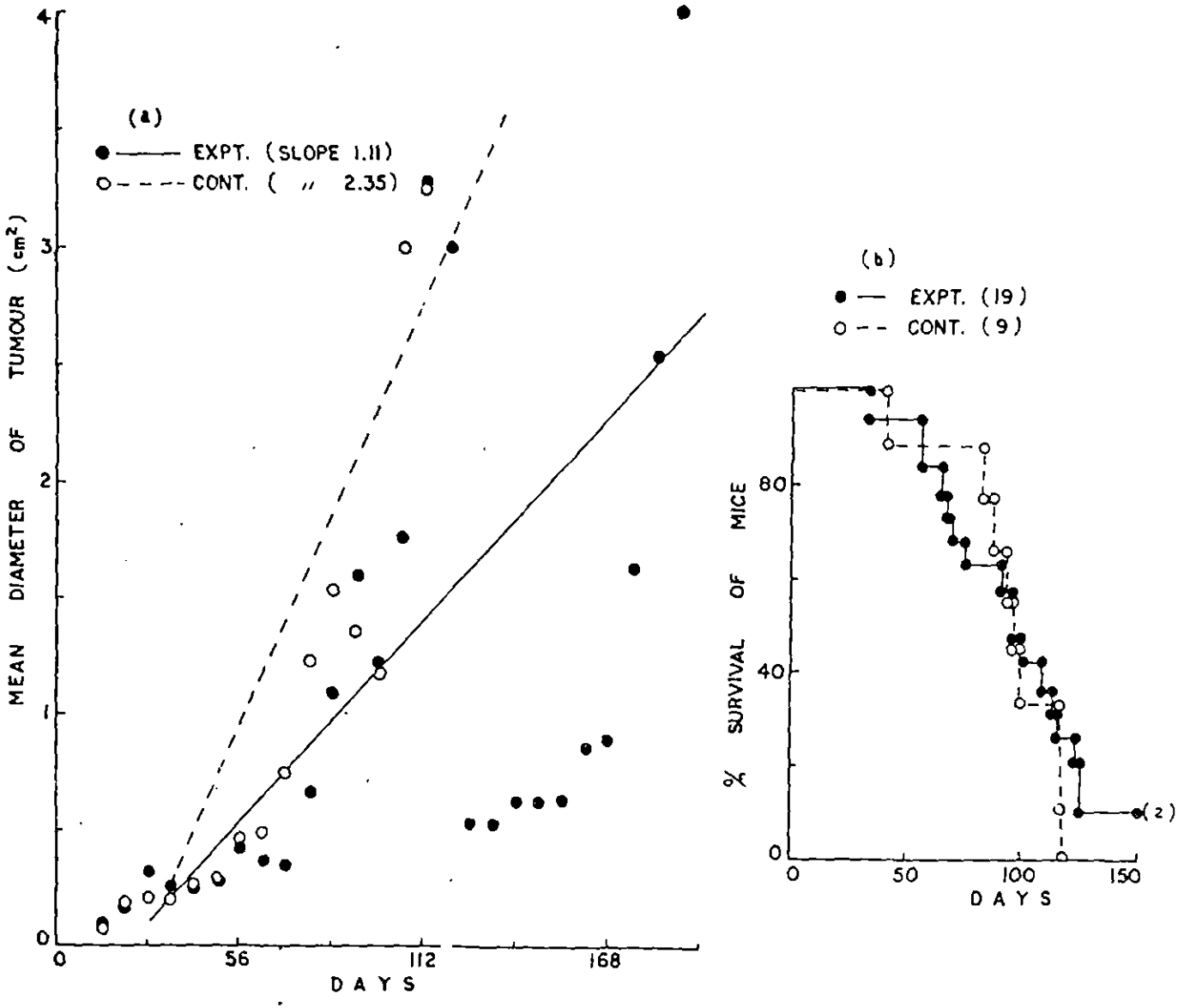

(a)
EXPT. (SLOPE 1.11)
CONT. (" 2.35)
MEAN DIAMETER OF TUMOUR (cm²)
0
1
2
3
4
0
56
112
168
DAYS
(b)
EXPT. (19)
CONT. (9)
% SURVIVAL OF MICE
0
40
80
0
50
100
150
DAYS
(2)

Figure 9a. Rate of tumour growth after repeated injections of Con A, measured every 7 days (day of MCA injection considered 0 day). The lines were drawn according to least square fit method. Values for Y upto 4.0 have been plotted in the figure.

Experimental (●--------) animals injected with Con A every 10 days for 12 times, Control (o--------) injected with normal saline following the same schedule.

b. Percentage of survival on different days of tumour bearing mice injected with Con A (Expt.) and normal saline (Cont.) following the above schedule.

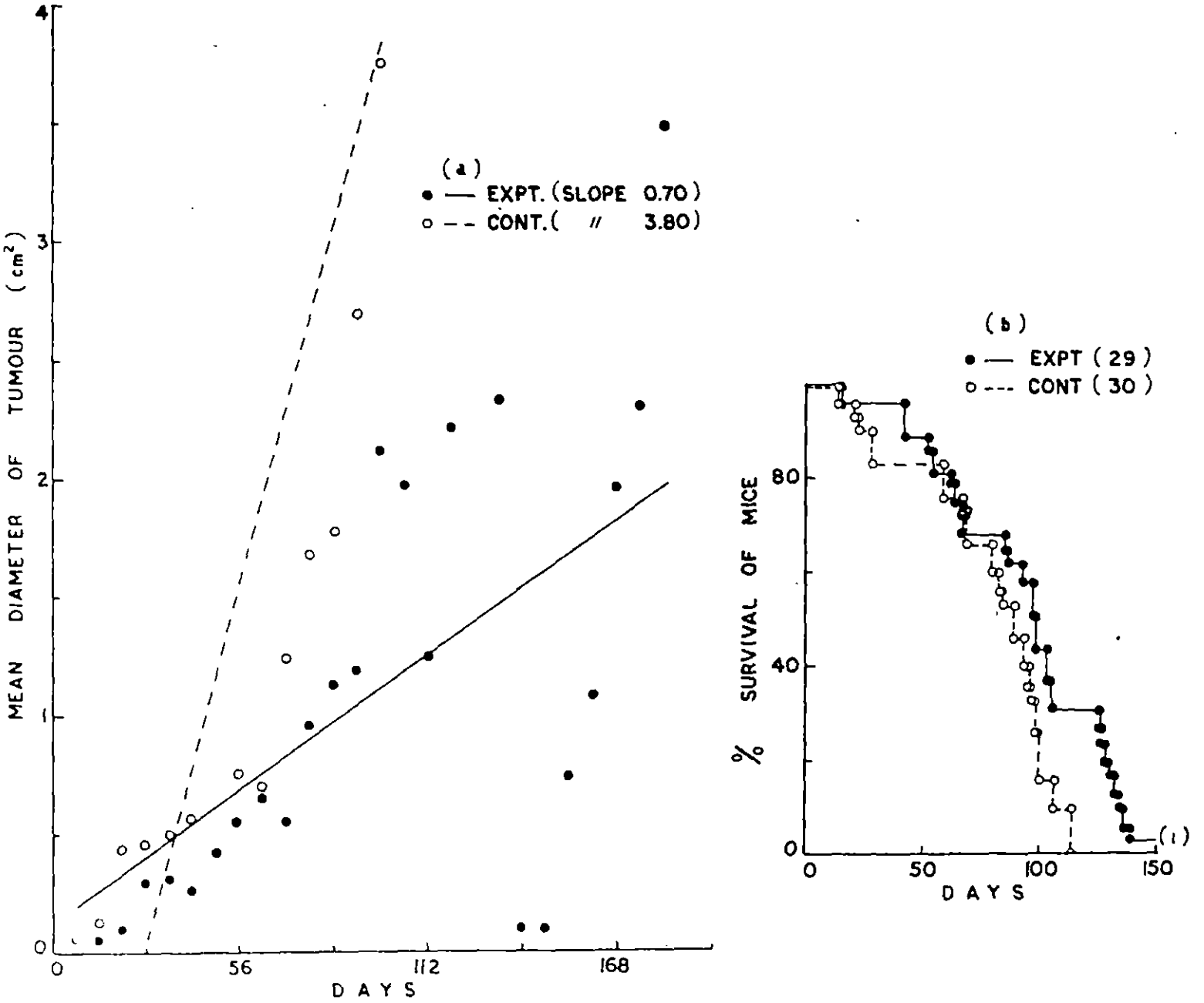

(a)
EXPT. (SLOPE 0.70)
CONT.(" 3.80)
MEAN DIAMETER OF TUMOUR (cm²)
DAYS
0
56
112
168
1
2
3
4
(b)
EXPT (29)
CONT (30)
% SURVIVAL OF MICE
DAYS
0
40
80
50
100
150

Figure 10. Repeatebility of blast induction by Con A in different lymphoid organs with repeated injection of Con A (50 µg/animal) at every 10 days for five times. Arrows indicate the day of Con A injections.

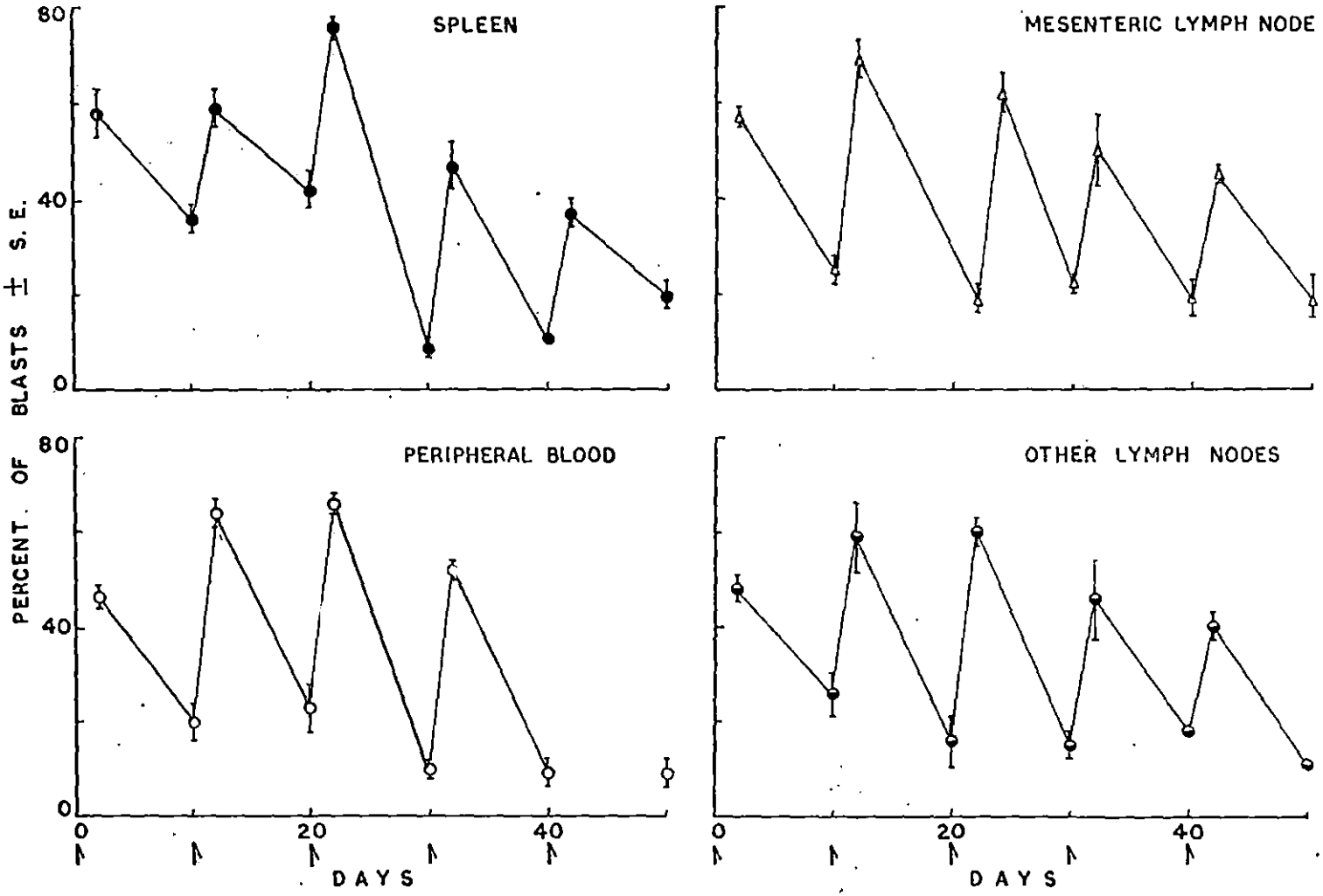

SPLEEN
MESENTERIC LYMPH NODE
PERIPHERAL BLOOD
OTHER LYMPH NODES
PERCENT OF BLASTS ± S.E.
80
40
0
0
20
40
DAYS

Figure 11. Average number of lymphocytes ± S.E. per unit area ($0.004mm^2$) observed in histological preparations of lymphoid organs from animals undergoing repeated injections of Con A (50 µg/animal) at every 10 days for 5 times.

a) Spleen and b) Lymph node. Arrows indicate the day of Con A injections.

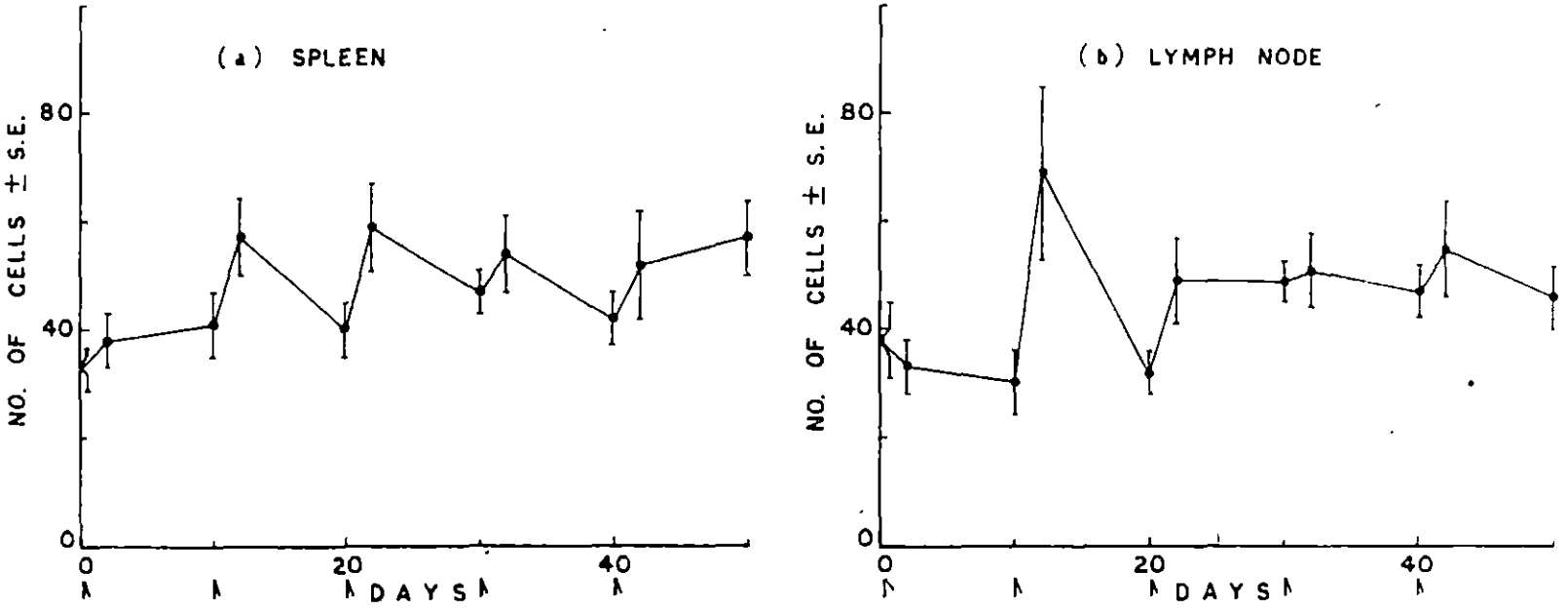

(a) SPLEEN
NO. OF CELLS ± S.E.
80
40
0
0
20
40
DAYS
(b) LYMPH NODE
NO. OF CELLS ± S.E.
80
40
0
0
20
40
DAYS

Figure 12a. Percentage of blasts generated at 48 hr with *in vivo* Con A (50 µg/animal) treatment in animals pretreated with different doses of cyclophosphamide.

b. Percentage of blasts generated *in vitro* at 48 hr with Con A (5 µg/ml) treatment of lymphocytes from the animals pretreated with different doses of cyclophosphamide.
[Con A stimulation was done 48 hr after cyclophosphamide treatment (i.p.)]

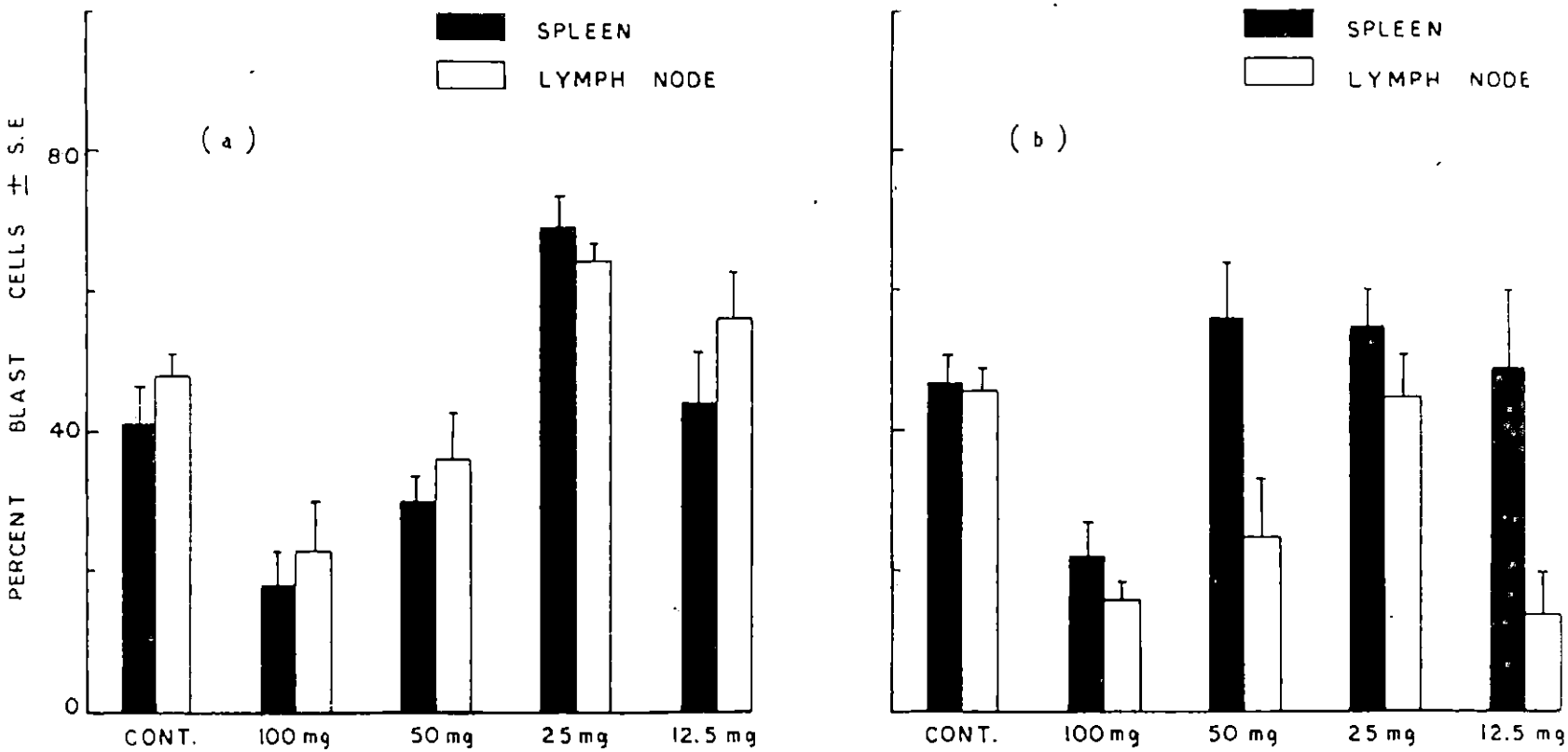
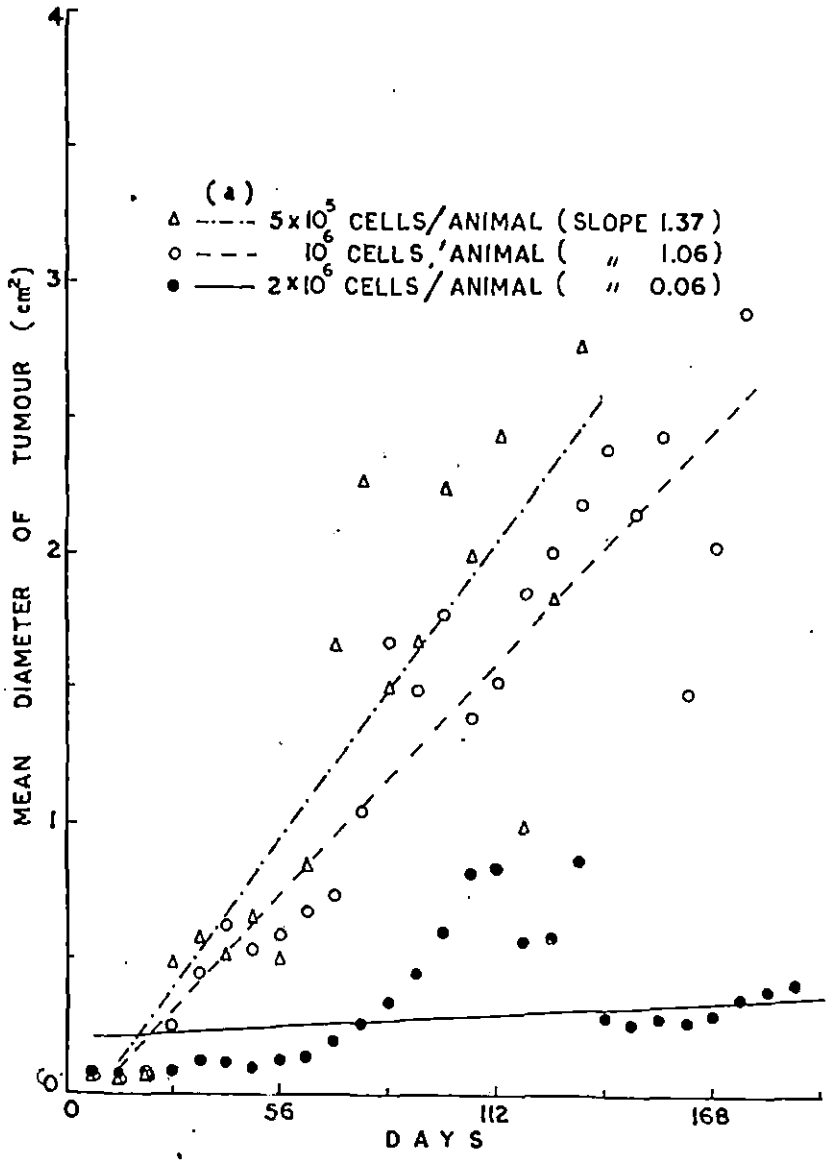

SPLEEN
LYMPH NODE
(a)
(b)
PERCENT BLAST CELLS ± S.E
80
40
0
CONT.
100 mg
50 mg
25 mg
12.5 mg

Figure 13a. Rate of tumour growth after injection of three different doses of activated lymphocytes, depleted of suppressor T cells by pretreatment of cyclophosphamide (25 mg/kg/animal, injected i.p. 48 hr prior to <u>in vivo</u> activation with Con A). Activated lymphocytes, collected 48 hr after Con A stimulation and injected at the tumour site on every 10th day for 16 times. Size of the tumours measured every 7 days (day of MCA injection considered 0 day).

The lines for tumour growth were drawn according to least square fit method and slopes were calculated. Values for Y upto 4.0 have been plotted in the figure. 5×10^5 cells/animal (Δ- - - - - Δ), 10^6 cells/animal (o- - - - -) and 2×10^6 cells/animal (• ———).

b. Percentage of survival of tumour bearing mice injected at the site of tumour with 5×10^5 (Δ- - - - - - -), 10^6 (o- - - - - - -) and 2×10^6 (• ———) activated lymphocytes depleted of T suppressor cells in the above schedule. Twelve animals constituted each experimental group and the number at the end of the plotting indicates the number of animals at that point.


(a)
Δ ---- 5x10^5 CELLS/ANIMAL (SLOPE 1.37)
○ - - - 10^6 CELLS/ANIMAL (" 1.06)
● —— 2x10^6 CELLS/ANIMAL (" 0.06)
MEAN DIAMETER OF TUMOUR (cm²)
0
1
2
3
4
0
56
112
168
DAYS

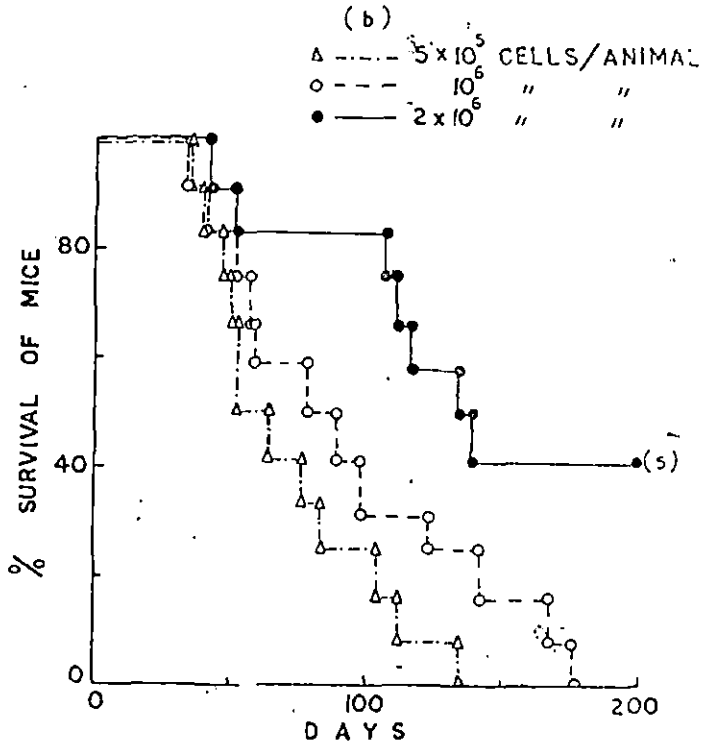

(b)
Δ ---- 5x10^5 CELLS/ANIMAL
○ - - - 10^6 " "
● —— 2x10^6 " "
% SURVIVAL OF MICE
0
40
80
0
100
200
DAYS
(s)

Table 1. Percentage of occurrence and time required for appearance of tumour (latency period) and life span (survivality) of tumour bearing mice after injection with three different doses of 3'-Methylcholanthrene of two brands.

Brand of MCA	Doses of MCA (mg/Animal)	Percentage[a] of appearance of tumour	Mean latency[b] period for appearance (Days ± S.E)	Mean survival of hosts (Days ± S.E)
Koch -Light Lab. Ltd., Colnbrook U.K.	0.5	100	32 ± 4	109 ± 9
	1.0	73	38 ± 4	92 ± 6
	2.0	84	42 ± 6	105 ± 8
Sigma Chem. Co., St. Louis, U.S.A.	0.5	75	57 ± 14	125 ± 20
	1.0	80	26 ± 3	117 ± 10
	2.0	89	15 ± 1	90 ± 5

a) Number of animals in different groups was from 8 to 19

b) Day of MCA injection considered 0 day.

Table 2. Efficacy of anti-mouse thymocyte serum (ATS) in the complement mediated killing of the lymphocytes from thymus and lymph nodes.

Type of cell	Expt. No.	LYTIC INDEX[a] WITH DIFFERENT DILUTION OF SERUM				
		1/10	1/20	1/40	1/80	1/160
Thymus	1	100	79	80	60	14
	2	99	80	60	59	4
	3	92	100	62	46	16
Lymph node	1	76	48	50	32	4
	2	72	58	31	22	4
	3	68	54	32	20	13

a) Lytic index = $\frac{\text{No. of living cells with normal serum} - \text{No. of living cells with ATS}}{\text{No. of living cells at the beginning in each tube}} \times 100$

Table 3. Blastogenesis induced with Con A in normal and T cell depleted animals.

Organ	PERCENT OF BLASTS ± S.E.		
	Normal animals	T cell depleted[a] animals	w/o Con A (control)
Spleen	57± 3	16± 1	9± 0.8
Mesenteric lymph node	54± 3	18± 3	6± 0.7

a) For depletion of T cells, animals were neonatally thymectomized and followed by anti - thymocyte serum treatment.

Table 4. Degree of different angiogenic reactions and occurrence of tumour in different combinations of grafts in the anterior eye chamber of mice.

Group	Types of grafts	No of animals	DEGREE OF REACTIONS ON DIFFERENT DAYS AFTER GRAFTING (Percentage of cases with initial reaction)[a]												Percentage of tumour occurrence
			Vasodilatation				Neovascular sprouting Primary		Secondary		Growth of grafts				
			Day: 4	8	12	25	4	8	12	25	4	8	12	25	
A	Tumour only	31	++ (90)	+++	++	+	++ (90)	++	++ (83)	+	+ (87)	+	+++	+++	74
B	Tumour + stimuted lymph node	33	+ (66)	±	±	-	+ (57)	±	- (66)	-	± (81)	±	±	-	15
C	Tumour + normal lymph node	29	++ (86)	+++	++	+	++ (86)	++	++ (79)	±	+ (89)	++	++	+	58

a) Percentages (in parentheses) have been calculated in reference to the reaction on the 4th day in all cases except for secondary neo-vascular reaction where the reaction on 12th day has been considered.

Table 5. Degree of angiogenic reactions and occurrence of tumour in presence of T cell depleted, Con A treated lymph node piece.

Group	Types of grafts	No. of animals	DEGREE OF REACTIONS ON DIFFERENT DAYS AFTER GRAFTING (Percentage of cases with initial reaction)[a]												Percentage of tumour occurrence
			Vasodilatation Days:				Neovascular sprouting Primary		Secondary		Growth of grafts				
			4	8	12	25	4	8	12	25	4	8	12	25	
A	Tumour + stimulated lymph node	12	+ (66)	±	±	-	+ (50)	±	- (75)	-	+ (75)	±	-	-	16
B	Tumour + stimulated lymph node depleted of T cells[b]	16	++ (87)	+++	++	++	+++ (50)	++	++ (81)	++	+ (93)	+++	+++	+++	85
C	Tumour only	26	++ (73)	+++	++	+	++ (73)	+	++ (73)	+	+ (92)	+	++	++	76

a) As in Table 4.

b) For depletion of T cells prior to Con A mediated stimulation, animals were neonatally thymectomized and followed by anti-thymocyte serum treatment as described in Materials and Method.

Table 6. Degree of different angiogenic reactions and occurrence of tumour from the tumour pieces transplanted in the anterior eye chamber of mouse after incubation with activated and normal non-activated lymphocytes.

Group	Tumour pieces incubated with	Time of incubation (hr)	No. of animals	DEGREE OF DIFFERENT REACTIONS[a] AFTER GRAFTING: Vasodilatation Day: 4	8	12	25	Neovascular sprouting Primary 4	8	Secondary 12	25	Degree of growth of tumour grafts on Day: 4	8	12	25	% of tumour occurrence
A	Activated lymphocytes	24	10	± (50)	-	-	-	- (50)	-	- (70)	-	± (50)	±	-	-	0
		48	24	± (54)	±	±	±	± (58)	±	± (45)	±	± (70)	±	±	±	8
B	Normal lymphocytes	24	10	++ (70)	++	+	±	++ (40)	+	++ (44)	±	+ (70)	+	++	++	62
		48	26	+ (61)	±	+	+	+ (76)	±	+ (63)	±	± (65)	±	+	++	50
C	(Control) non-incubated fresh tumour piece	-	27	++ (88)	+++	++	+	++ (88)	++	++ (81)	+	++ (85)	++	++	++	70

a) As in Table 4.

Table 7. Measure of body weight and secondary lymphoid organs' weight and number of viable lymphocytes in the organs and in peripheral blood during repeated *in vivo* stimulation with Con A.

Days	Mean body weight of the animals ± S.E. (gm)	Spleen		Mesenteric lymph node		Other lymph nodes[b]		Peripheral blood
		Mean weight ± S.E. (mg)	No.of viable lymphocytes ($\times 10^7$)	Mean weight ± S.E. (mg)	No.of viable lymphocytes ($\times 10^7$)	Mean weight ± S.E. (mg)	No. of viable lymphocytes ($\times 10^7$)	No. of viable lymphocytes (per ml) ($\times 10^7$)
0[a]	20.7±0.6	80±0.6	4.8	58± 1.4	3.9	24±1.1	1.2	1.1
10	21.1±17	78±3.4	4.9	54± 1.2	2.5	21±2.3	1.6	1.3
30	30.5±.75	102± 6.4	5.3	57± 3.5	3.3	24± .6	1.7	0.9
50	29[c] ± 1.2	94±22.2	4.1	49±14.0	2.7	25±3.9	1.2	1.3

a) Weight and other indices of the normal animal of 6 weeks of age at 0 day of the experiment.

b) Five other regional lymph nodes pooled together.

c) Average body weight of normal mice of same age was 31 gm.

Table 8. Degree of precipitation[a] obtained by immunodiffusion in Ouchterloni plate of serum from animals injected repeatedly[b] with Con A and fresh Con A solution (50 µg/0.1 ml) in the central well.

Days[c] of serum collection	DILUTIONS OF SERUM										
	Expt.1						Expt.2				
	1:1	1:2	1:4	1:8	1:16	1:32	1:10	1:20	1:40	1:80	1:160
0 (Control)	-	-	-	-	-	-	-	-	-	-	-
30	+++	+++	++	+	+	-	+	±	-	-	-
50		+++	++	+	+	-	+	±	-	-	-

a. Reactions of a representative experiments have been shown.

b. Injected with 50 µg Con A/animal at 10 days intervals upto 5 times.

c. Serum was collected before the 1st injection (control) and 10 days after each of 3rd and 5th injection.

Table 9. Con A activated lymphocytes mediated killing of MCA-induced tumour target cells in ^{51}Cr release assay.

Activation schedule	Expt. No.	Effector cells from:	PERCENT CYTOTOXICITY + S.E. at different target : effector cells											
		Spleen						Lymph node						
		1:200	1:100	1:50	1:10	1:5	1:1	1:200	1:100	1:50	1:10	1:5	1:1
50 µg/Con A/ animal for 24 hr	I	18±5.5	30±8.6	20±2.6	31±2.8	23±6.3		18±2.0	24±5.1	27±1.0	33±4.3	24±5.3	
	II	22±4.4	18±0.3	18±0.5	15±1.2	10±1.5		26±4.5	15±1.8	18±1.0	12±0.6	15±1.2	
50 µg Con A/ animal for 48 hr	I	50±2.6	11±3.3	21±5.0				-	-	-	-		
	II		38±1.5	48±11.6	33±2.1				30±1.8	44±2.4	39±1.2		
	III	52±0.6	53±2.5	60±1.5	53±6.1			54±7.5	62±3.1	66±2.5	56±2.5		
	IV	41±8.0	14±0.0	23±4.9	10±2.9	6±5.3		21±4.7	31±13.5	18±3.1	3±5.3	14±0.6	
	V	29±1.7	47±6.6	58±00	34±9.2	18±7.5	8±2.8	25±1.5	44±5.6	53±5.8	28±3.6	4±2.0	4±4.0

/Contd.....

Table 9. (Contd.)

Activation schedule	Expt. No.	PERCENT CYTOTOXICITY + S.E. at different target : effector cells											
		Effector cells from : Spleen						Lymph node					
		1:200	1:100	1:50	1:10	1:5	1:1	1:200	1:100	1:50	1:10	1:5	1:1
No activation (Control)	I	-10±4.9	0±1.7	3±0.5				-17±19.2	0.5±2.8				
	II	13±2.0	12±0.8	14±0.3	14±0.3	16±0.6		13±0.6	14±0.5	13±0.3	11±1.1	12±0.6	
	III	4±4.1	14±2.3	-1±8.7	14±27.0	-2±13.5		14±5.4	7±13.5	19±18.2	19±4.1	11±11.8	
	IV	12±1.7	6±2.8	13±1.7	-1±0.4	-3±1.2		7±3.5	7±4.8	11±4.9	1.7±3.4	-5±1.2	
50 µg Con A/ animal depleted of T cells *	I	-4	-7	-1	-3				-22	0.7	-2		
	II	-1±7.5	3±3.6	7±7.0	1±4.0				6±2.6	2±4.2	-1±3.6		

*For depletion of T cells, animals were neonatally thymectomized and followed by anti-thymocyte serum treatment as in Materials and Methods.

Table 10. Susceptibility of Con A transformed blasts and small lymphocytes to anti-thymocyte serum.

Expt. No.	Source of cells	Serum	Types of cells	PERCENTAGE OF LYSIS OF CELL NUMBER							
				Dilutions of serum							
				1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
1.	Spleen	ATS	Blasts	89	98	81	80	77	72	-	-
			Small lymphocytes	35	73	53	30	-9	4	-	-
		NRS[a]	Blasts	-3[b]	15	0	16	17	5	-	-
			Small lymphocytes	-18	0	-10	-2	-9	-1	-	-
	Lymph node	ATS	Blasts	99	98	87	85	79	78	-	-
			Small lymphocytes	85	70	65	42	18	9	-	-
		NRS	Blasts	9	7	19	7	2	4	-	-
			Small lymphocytes	17	-10	-7	1	0	1	-	-

/Contd.....

Table 10 (Contd.)

Expt. No.	Source of cells	Serum	Types of cells	PERCENTAGE OF LYSIS OF CELL NUMBER							
				Dilutions of serum							
				1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
2.	Spleen	ATS	Blasts	82	87	84	76	58	31	7	13
			Small lymphocytes	14	18	11	-10	-2	-5	-8	-9
		NRS	Blasts	13	2	6	24	14	16	6	4
			Small lymphocytes	4	-2	-8	-3	2	-17	-6	-4
	Lymph node	ATS	Blasts	80	95	82	80	87	49	12	12
			Small lymphocytes	24	38	14	-8	-2	-12	11	3
		NRS	Blasts	23	2	17	5	22	13	13	18
			Small lymphocytes	12	-2	-15	5	-7	-19	-22	-12

a) Control with Normal Rabbit Serum (NRS)

b) Negative values were due to a few extra cell encountered in the tube than average initial number seeded in each tube. For practical reason this index can be considered as zero.

Table 11. Change in weight of secondary lymphoid organs after 48 hr of treatment with different doses of cyclophosphamide (Cy) and subsequent intravenous injection of Con A.

Dose of Cy	48 hr after Cy treatment			48 hr after Con A[a] treatment		
	Mean wt[b] (mg) ± S.E. of			Mean wt[c] (mg) ± S.E. of		
	Spleen	Mesenteric lymph node	Other lymph nodes[d]	Spleen	Mesenteric lymph node	Other lymph nodes[d]
100 mg/kg	27 ± 2.2.	23 ± 2.1	12 ± 1.8	39 ± 3.0	35 ± 3.3	14 ± 1.4
50 mg/kg	45 ± 11.5	43 ± 8.0	16 ± 2.2	47 ± 3.6	41 ± 5.1	18 ± 1.7
25 mg/kg	62 ± 2.7	47 ± 8.1	20 ± 1.6	88 ± 8.4	70 ± 7.9	31 ± 2.6
12.5 mg/kg	59 ± 12.0	62 ± 21.2	17 ± 5.3	75 ± 12.9	86 ± 10.5	30 ± 9.6
(Control) 0.5 ml Dist.Water	82 ± 15.1	62 ± 14.7	26 ± 2.5	97 ± 17.6	69 ± 9.0	29 ± 4.7

a. 50 µg of Con A/animal intravenously injected 48 hr after Cy treatment

b. Mean values from four animals. c. Mean values from six animals.

d. Five lymph nodes taken together from pooled maxillary, cervival, axillary and inguinal lymph nodes.

Table 12. Incorporation of ^{3}H-TdR by the lymphocytes activated *in vivo* with Con A after treatment with different doses of cyclophosphamide (Cy).

Expt. No.	Dose of Cy[a]	Animal No.	Spleenocytes CPM for replicates[b]			Spleenocytes Mean ± S.E.	Lymph node cells CPM for replicates			Lymph node cells Mean ± S.E.
1.	100 mg/kg	1	2697	3663	3630		2090	1740	4129	
		2	1842	3288	2108	2188 ± 401	1105	690	652	1306 ± 406
		3	629	1137	704		254	570	531	
	50 mg/kg	1	652	794	1380		708	770	1188	
		2	8206	15403	8705	3256* ± 1018	2131	2628	2160	1285 ± 234
		3	2922	2309	2793		-	559	737	
	25 mg/kg	1	2242	1909	3837		-	-	-	
		2	4421	6247	5147	5016* ± 826	2153	5962	5458	4524* ± 1184
		3	7819	5310	-		-	-	-	
	(Control) w/o Cy[c]	1	890	877	1763		1632	2962	1367	
		2	689	1251	1026	1120 ± 113	988	666	713	1710 ± 488
		3	889	1471	1226		2697	2148	2219	

/Contd....

Table 12 (Contd.)

Expt. No.	Dose of Cy[a]	Animal No.	Spleenocytes CPM for replicates[b]			Mean ± S.E.	Lymph node cells CPM for replicates			Mean ± S.E.
2.	100 mg/kg	1	1082	1580	884		943	399	558	
		2	870	431	500	1338 ± 291	762	375	375	450* ± 260
		3	1734	1688	3276		226	141	274	
	50 mg/kg	1	3179	3127	1893		592	569	350	
		2	591	446	1149	1730 ± 494	201	180	180	345 ± 78
		3	-	-	-		-	-	-	
	25 mg/kg	1	2262	4645	3695		845	1238	602	
		2	2056	1549	2504	3535* ± 662	570	634	1254	857 ± 129
		3	5283	6918	4106		-	-	-	
	12.5 mg/kg	1	3306	3698	4378		1270	1471	860	
		2	7080	6676	5227	4510* ± 502	396	1306	2101	1055 ± 178
		3	3656	3809	2768		856	735	506	
	(Control)	1	1901	1057	561		279	264	1316	
	w/o Cy[c]	2	4612	2458	-	2683 ± 806	692	858	1032	1118 ± 273
		3	5512	1242	-		2284	2588	783	

a Different doses of cyclophosphamide in 0.5 ml of distilled water were given i.p. 48 hr prior to i.v. injection of Con A (50 µg/animal)

b Incubation in presence of ^{3}H-Thymidine for 12 hr in vivo

c 0.5 ml of distilled water without cyclophosphamide given i.p. 48 hr earlier to Con A injection.

Significance of the difference with control has been calculated by Student's t test.
p value equal to or less than 0.05 was considered signficant and indicated by *.

Table 13. Cytotoxic killing of MCA-induced tumour target cells by Con A activated lymphocytes obtained from animals pretreated with different doses of cyclophosphamide (Cy)

Dose of Cy[a]	Expt. No.	PERCENT CYTOTOXICITY ± S.E. at different target : effector cells					
		Effector cells[b] from : Spleen			Lymph node		
		1:100	1:50	1:10	1:100	1:50	1:10
50 mg of Cy/kg	I	48± 3.1	49± 3.0	13± 6.3	42± 1.6	47± 6.6	42± 8.3
	II	2± 4.6	3± 4.0	8± 4.6	24± 3.9	7± 2.9	5±13.0
	III	22± 4.3	32± 2.6	35± 1.4	41± 4.6	58± 9.8	30± 5.7
25 mg of Cy/kg	I	93± 12.5	70± 4.6	74± 8.1	67± 8.1	63± 1.6	67± 1.4
	II	52± 11.9	60± 7.9	25± 4.7	-	-	-
	III	92± 4.5	77± 2.6	72± 6.1	102± 3.9	104± 12.4	77± 8.1

/Contd....

Table 13. Contd.

Doses of Cy[a]	Expt. No.	PERCENT CYTOTOXICITY + S.E. at different target : effector cells — Effector cells[b] from : Spleen 1:100	Spleen 1:50	Spleen 1:10	Lymph node 1:100	Lymph node 1:50	Lymph node 1:10
12.5 mg of Cy/kg	I	81± 15.7	65± 4.9	75± 4.5	73± 0.8	56± 13.7	68± 2.5
	II	30± 2.6	17± 6.7	17± 8.9	25± 6.1	40± 19.2	19± 11.1
	III	30± 4.1	32± 3.5	26± 11.5	41± 27.8	44± 3.3	55± 11.0
(Control)	I	40± 2.3	45± 6.3	65± 2.4	49± 7.0	46± 8.6	53± 4.9
	II	26± 2.3	25± 0.9	25± 1.8	25± 3.0	28± 0.5	26± 1.5
	III	22± 6.1	25± 3.3	27± 2.3	27± 4.9	36± 8.6	4± 5.0

a Injected i.p. per animal prior to Con A (50 µg/animal, including control) injection

b Effector cells were collected after 48 hr of *in vivo* Con A stimulation.

PLATE 1

Diagrammatic representation of changes in angiogenesis and growth of the tumour grafts in the anterior eye chamber of mice. Chronological changes in different groups are indicated by the segments arranged in a clockwise fashion (thickness of blood vessels indicates different degrees of vasodilatation; different types of grafts are indicated by different shades as indexed in the middle of the figure).

Normal, showing arrangements of circular limbal and radiating corneal vessels in a normal eye without any graft.

A-Eye with tumour graft only- shows gradual thickening of blood vessels, primary sprouting of blood vessels on day 4, secondary sprouting on day 8 and capillary anastomosis on day 25 over the bulged our graft.

B-Eye with tumour graft plus stimulated lymph node piece showing feeble neo-vascular reactions (angiogenesis) and no tumour growth.

C-Eye with tumour graft with non-stimulated lymph node piece showing reactions similar to that of Con A.

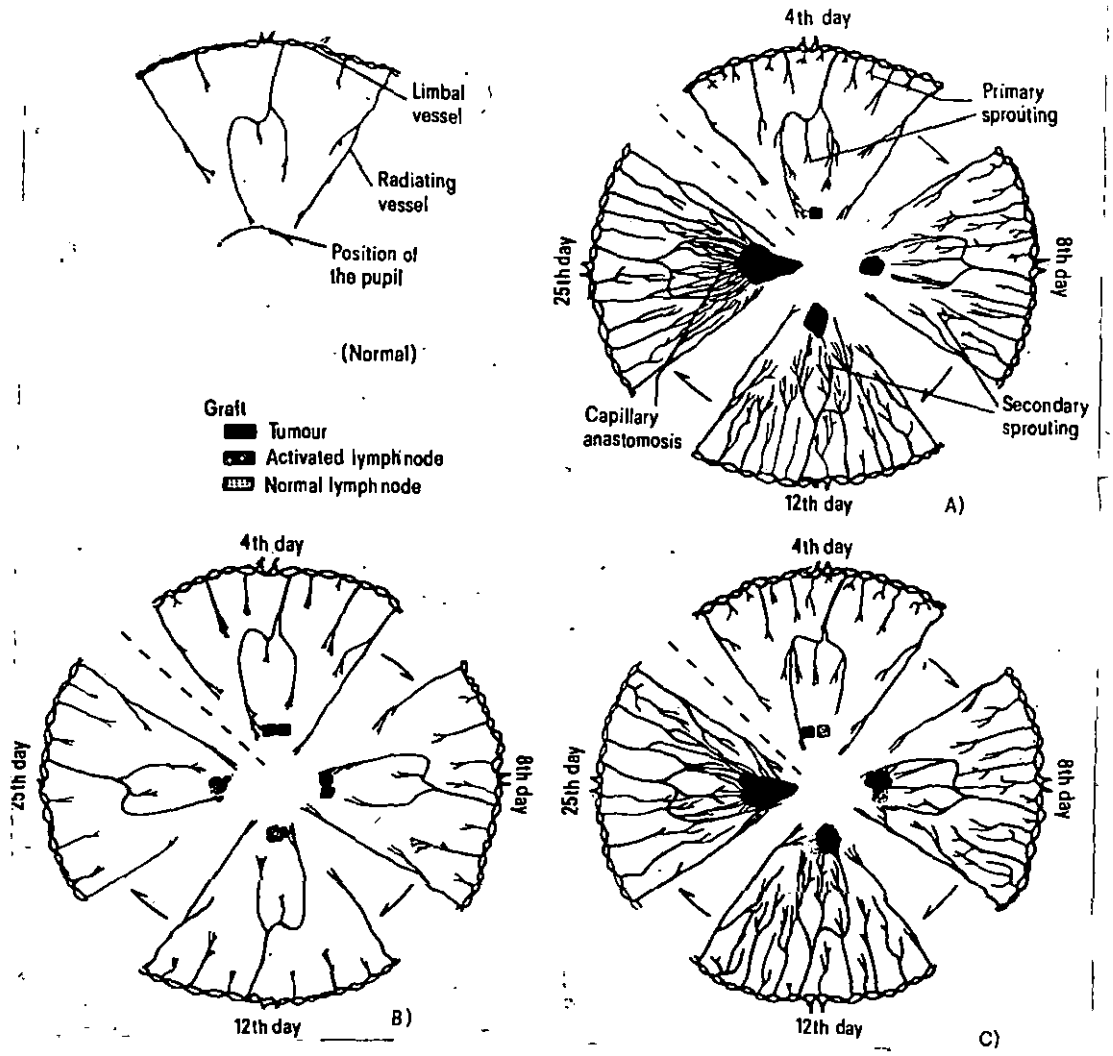

Limbal vessel
Radiating vessel
Position of the pupil
(Normal)
Graft
Tumour
Activated lymph node
Normal lymph node
4th day
Primary sprouting
25th day
8th day
Capillary anastomosis
Secondary sprouting
12th day
A)
4th day
25th day
8th day
12th day
B)
4th day
25th day
8th day
12th day
C)

PLATE 2

Photographs through dissection microscope of an eye of a mouse on different days after having a tumour piece transplanted in the anterior eye chamber.

Fig. 1. Primary angiogenic reactions on day 4, like dilatation of circular limbal vessels and appearance of profuse primary radial blood capillaries in cornea from the dilated vessels. X20.

Fig. 2. Elongation and bending of dilated radiating blood vessels towards the tumour graft(arrow) and appearance of secondary capillaries from the tips of the primary vessels on day 8. X50.

Fig. 3. Secondary angiogenic reaction on day 12. X15.

Fig. 4. On day 25 the outline blood vessels are obliterated due to rapid growth of the tumour transplant beyond the corneal limit. X20.


1


2


3

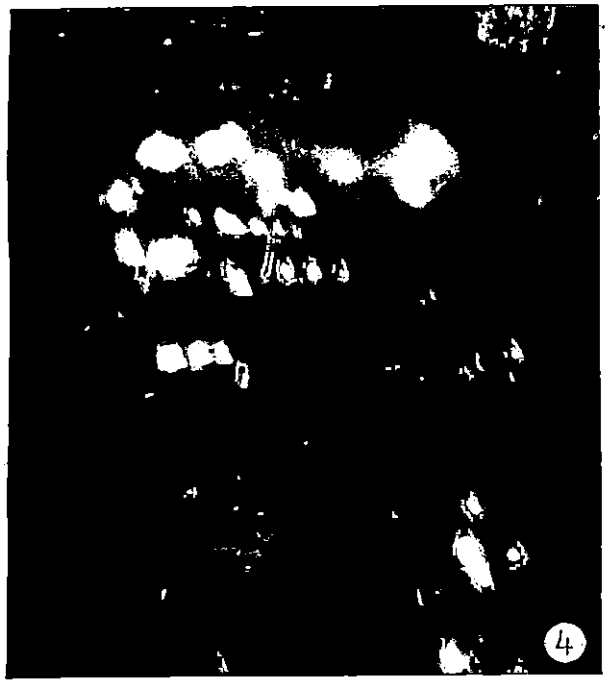

4

PLATE 3

Photographs through dissecting microscope showing the 8th day neo - vascular reactions in eyes carrying different combinations of grafts in the anterior eye chamber.

Fig. 1. Primary and secondary vascular reactions induced by a tumour piece in combination with a normal non-activated lymph node piece. X25.

Fig. 2. Very feeble or almost no angiogenic reaction induced by a tumour piece in combination with a Con A activated lymph node piece. X20.

Fig. 3. Enhanced vascular reactions induced by a tumour piece in combination with a Con A stimulated lymph node piece depleted of T cells. X50.


1


2


3

PLATE 4

Photographs through dissecting microscope of eye of mice having syngeneic normal and Con A stimulated lymph node piece transplanted in the anterior eye chamber.

Fig. 1. Arrangement of blood vessels almost similar to normal eye, 8 days after transplantation of normal, nonstimulated syngeneic lymph node piece. X15.

Fig. 2. Very feeble or almost no angiogenic reactions observed 8 days after transplantation of Con A stimulated syngeneic lymph node piece. X15.


1


2

PLATE 5

Diagrams represent summarily the condition of neovascular reactions on day 25 by different combinations of grafts in the anterior eye chamber of mice. Thickening of limbal vessels and radiating corneal vessels, primary and secondary sproutings from these vessels towards the tumour graft alone and tumour with normal lymph node. The reactions notably inhibited in presence of stimulated lymph node.

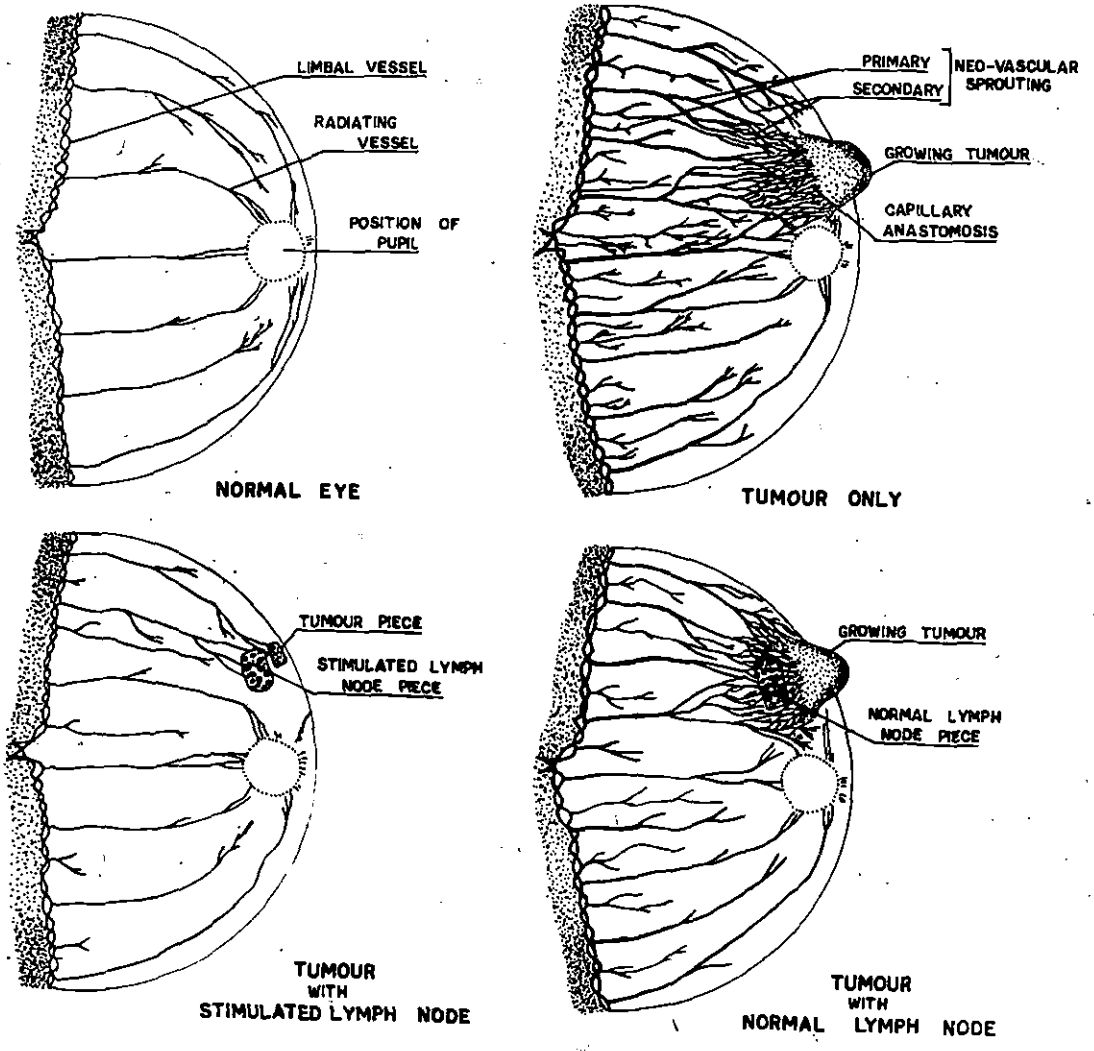

LIMBAL VESSEL
RADIATING VESSEL
POSITION OF PUPIL
NORMAL EYE
PRIMARY
SECONDARY
NEO-VASCULAR SPROUTING
GROWING TUMOUR
CAPILLARY ANASTOMOSIS
TUMOUR ONLY
TUMOUR PIECE
STIMULATED LYMPH NODE PIECE
TUMOUR WITH STIMULATED LYMPH NODE
GROWING TUMOUR
NORMAL LYMPH NODE PIECE
TUMOUR WITH NORMAL LYMPH NODE

PLATE 6

Photomicrographs of T.S. through spleen.

Fig. 1. Organisation of lymphoid tissue in normal spleen, without any germinal centre. X100.

Fig. 2. High power view of the same. X300.

Fig. 3. Histological organisation of the spleen after 48 hr of 2nd injection of Con A showing aggregation of lymphocytes into a germinal centre (GC) in between trabeculae (Tr). X125.

Fig. 4. High power view of the same. X400.

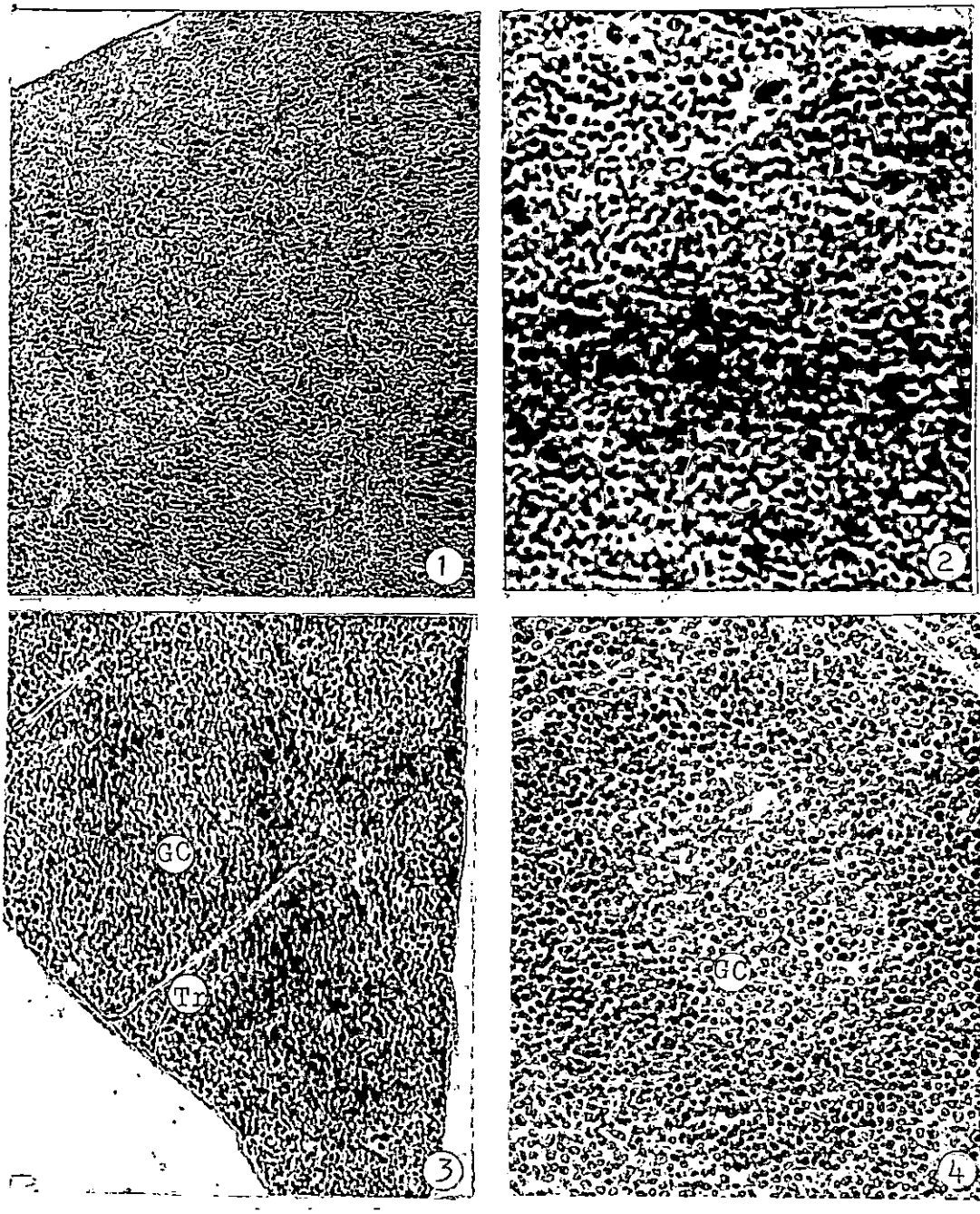

1
2
GC
Tr
3
GC
4

PLATE 7

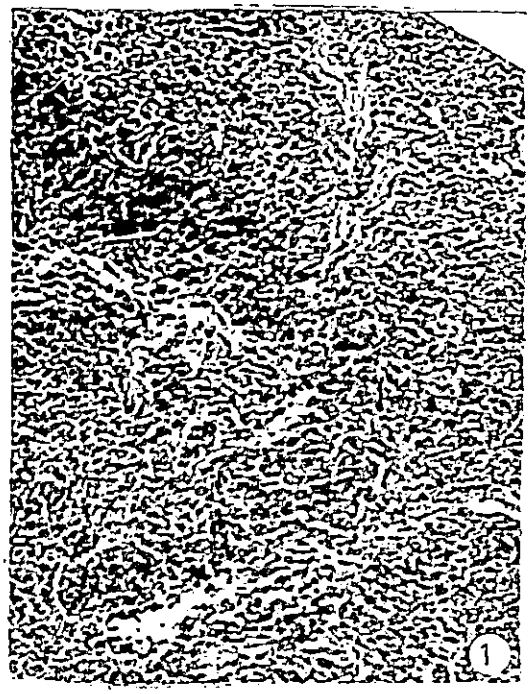
Photomicrographs of T.S. through spleen of mice injected repeatedly with 50 µg of Con A/animal.

Fig. 1. Histological organisation of spleen after 10 days of 2nd injection of Con A showing the appearance of gaps in lymphoid tissue. X150.

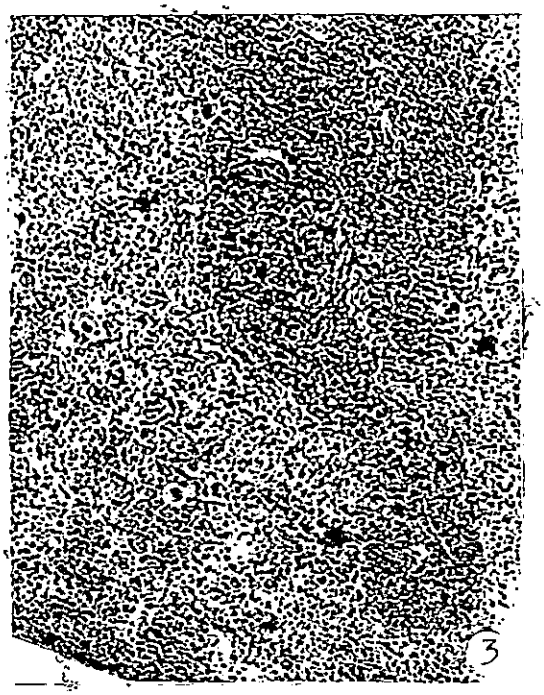
Fig. 2. High power view of the same. X400.

Fig. 3. Histological preparation of spleen after 48 hr of 3rd injection of Con A showing again the aggregation of lymphocytes in to germinal centres. X125.

Fig. 4. High power view of the same. X250.


1


2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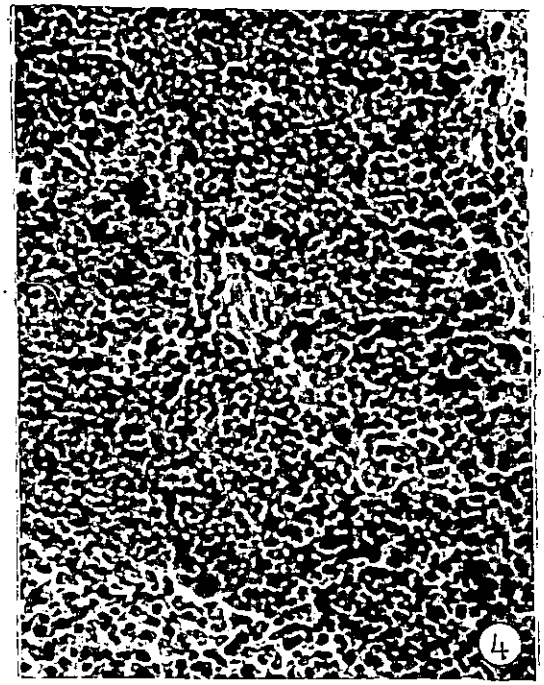

4

PLATE 8

Photomicrographs of T.S. through lymph node.

Fig. 1. Organisation of lymphocytes in normal organ. X100.

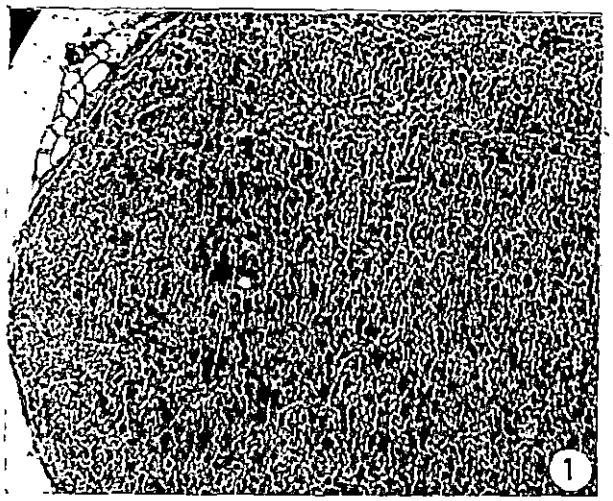
Fig. 2. High power view of the same. X500.

Fig. 3. Histological organisation of lymph node after 48 hr of 2nd injection of Con A showing aggregation of lymphocytes in to germinal centres (GC). X100.

Fig. 4. High power view of the same. X250.

Fig. 5. Enlargement of marginal sinus (MS) and appearance of medullary sinuses (MdS) after 10 days of 1st Con A stimulation. X100.

Fig. 6. High power view of the same. X250.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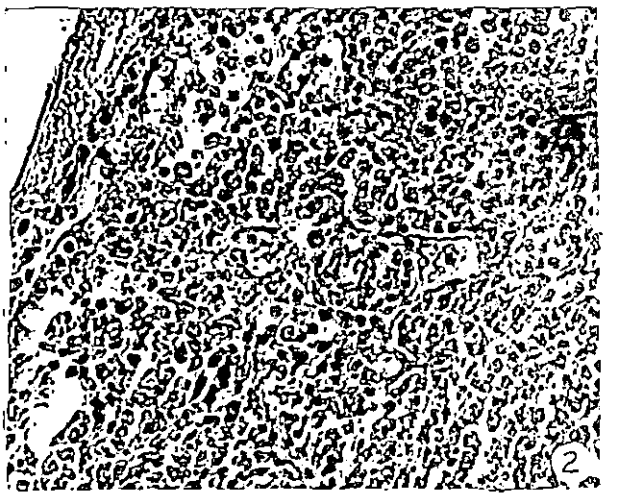
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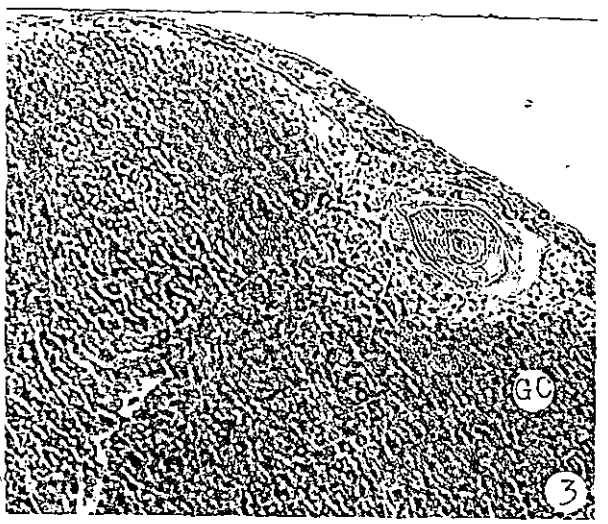
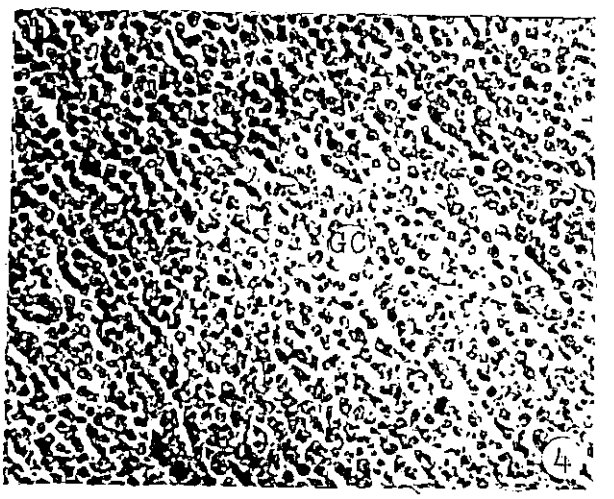
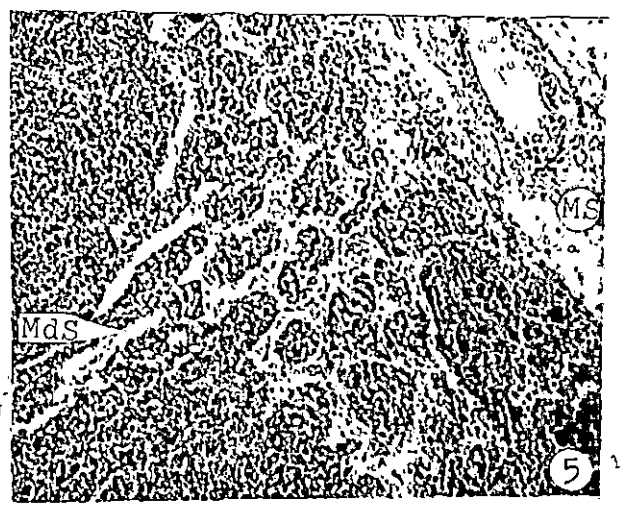

GC
3


GC
4


MS
MdS
5

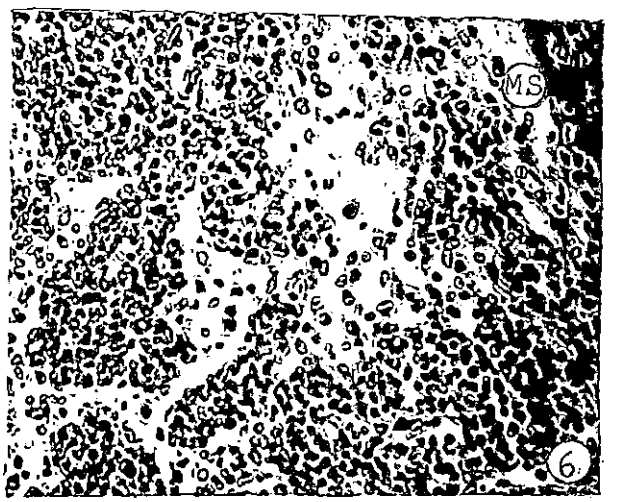
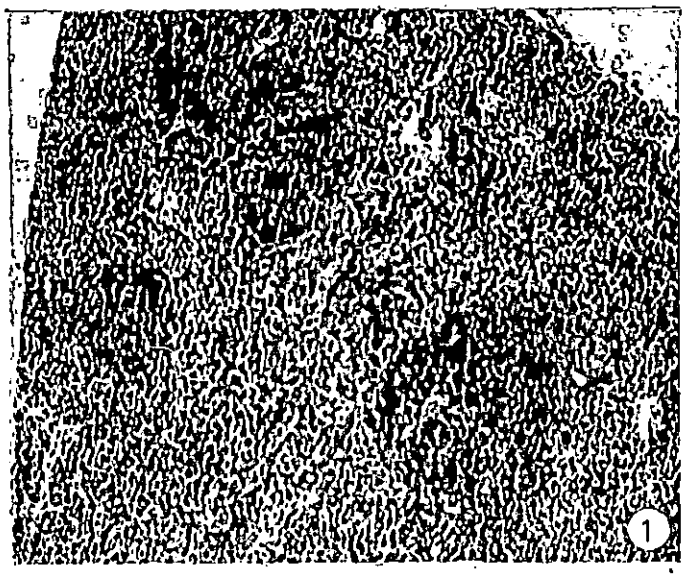

MS
6

PLATE 9

Photomicrograph of T.S. through (1) spleen (X125) and (2) lymph node (X100) after 48 hr of 5th injection of Con A showing the capability of lymphocytes to divide and aggregate in to germinal centres.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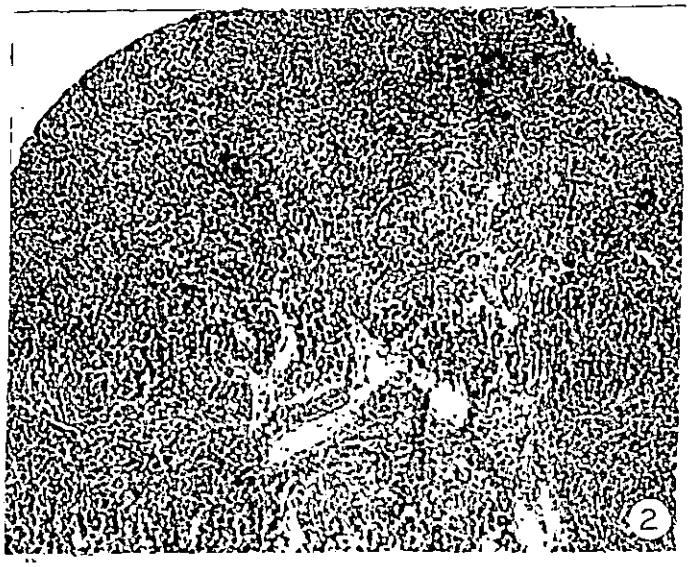
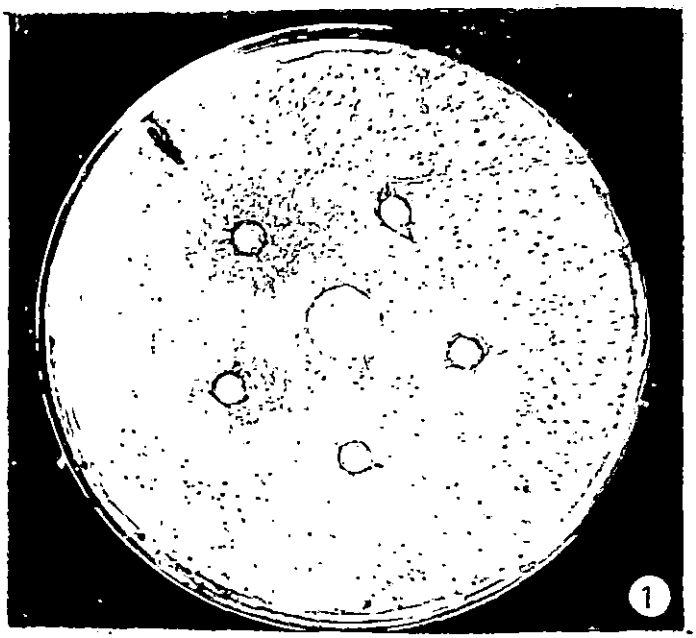

2

PLATE 10

Fig. 1. Photograph showing precipitation reaction of serum obtained from mouse after three injections of Con A with Con A solution in central well of Ouchterlony plate. In this experiment, peripheral wells contained serial two fold dilutions of serum starting from 1:1 (in marked well) in anti-clockwise direction.

Fig. 2. Control - Serum from normal animal without any injection of Con A did not cause any precipitation reaction. Content of central well and dilutions of serum in peripheral wells was as in Fig. 1.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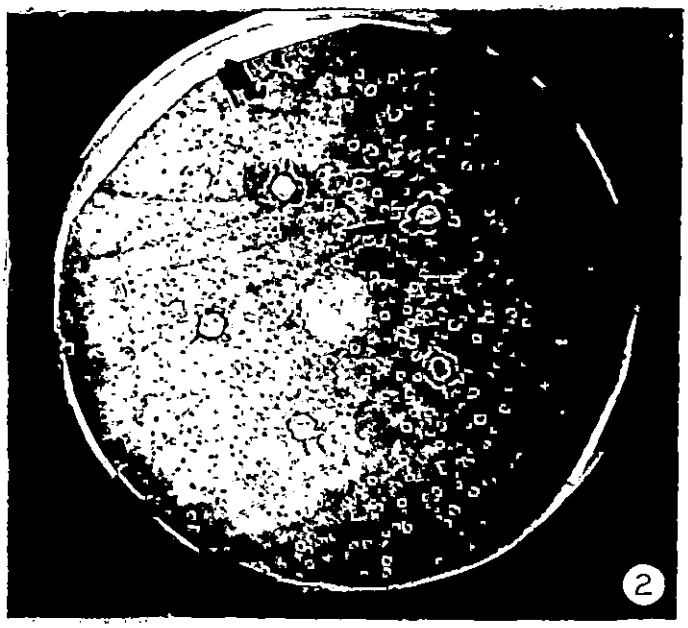
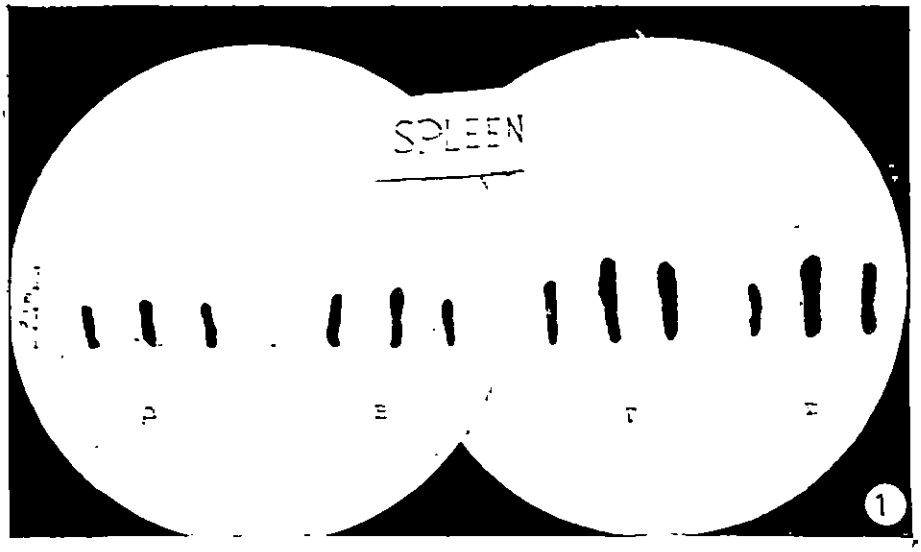

2

PLATE 11

Fig. 1. Photograph showing the reduction in size of spleen after treatment with different doses of cyclophosphamide.
A 100 mg, B 50 mg, C 25 mg/kg of body wt/animal and D Control without cyclophosphamide treatment.

Fig. 2. Photograph showing increment in size of spleen after intravenous Con A injection in animals pretreated with A 100mg, B 50mg, C 25mg, D 12.5mg of cyclophosphamide per kg of body wt/animal and E Control.


SPLEEN
1

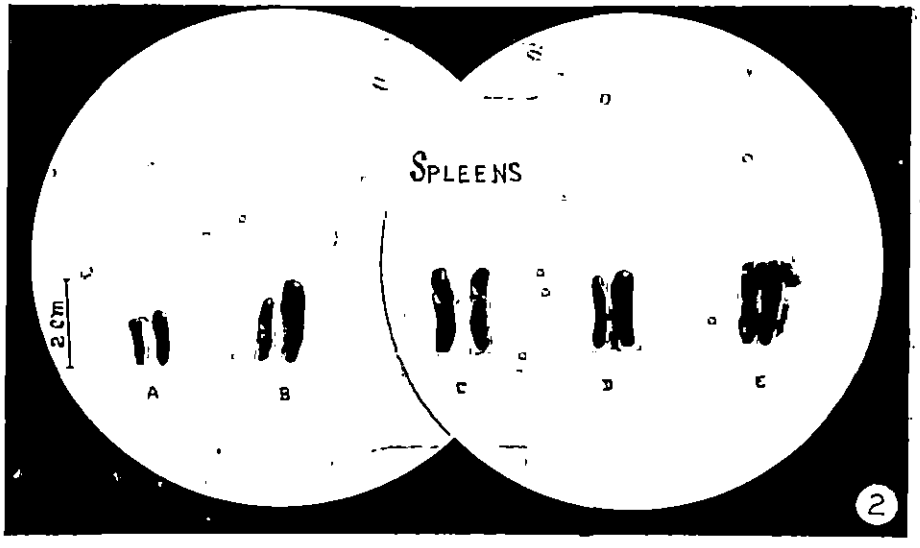
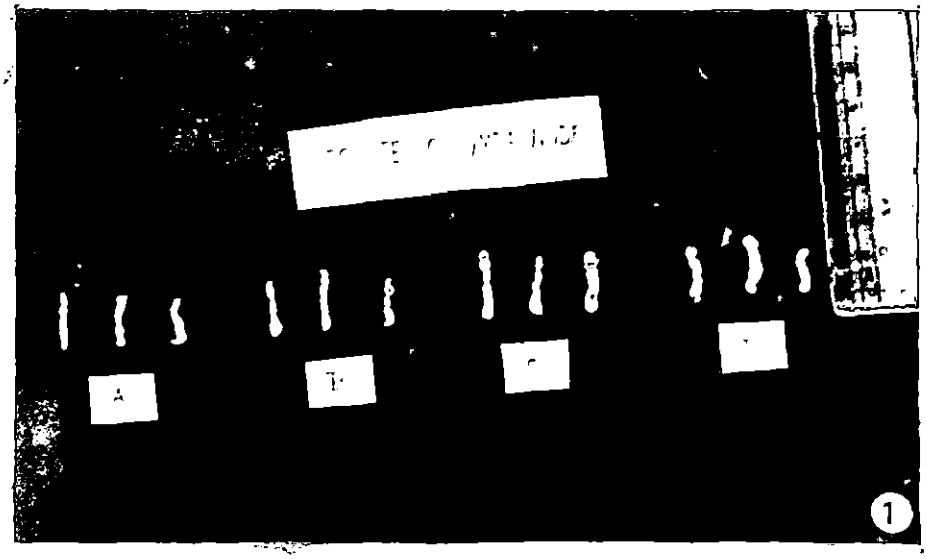
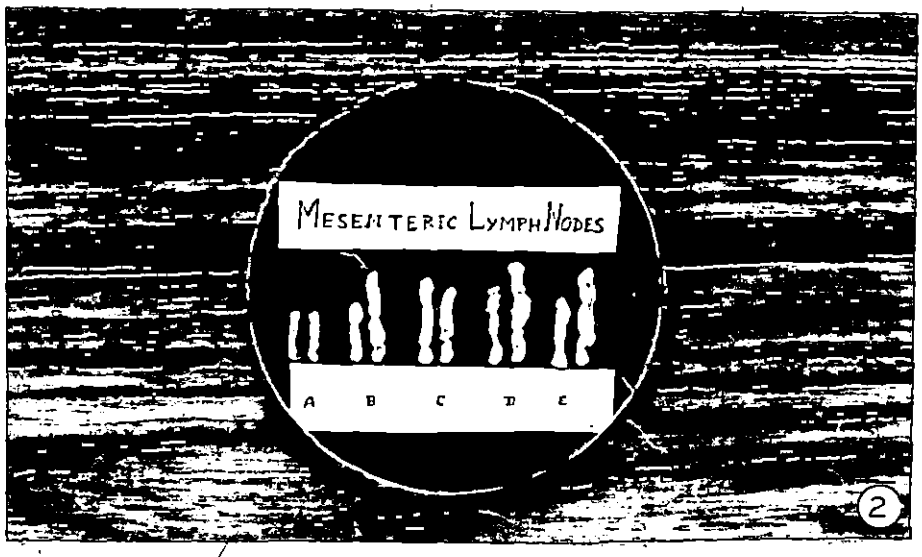

SPLEENS
2 cm
A
B
C
D
E
2

PLATE 12

Fig. 1. Photograph showing the reduction in size of mesenteric lymph node after treatment with different doses of cyclophosphamide. A 100mg, B 50mg, C 25mg of cyclophosphamide per kg of body wt/animal and D control without cyclophosphamide treatment.

Fig. 2. Photograph showing increase in size of mesenteric lymph node in animals pretreated with A 100mg, B 50mg, C 25mg, D 12.5mg of cyclophosphamide/kg of body wt/animal and E Control, and then injected with Con A.


A
1


MESENTERIC LYMPH NODES
A B C D E
2

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