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).