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Polyclonally stimulated murine T cells in control of fibrosarcoma *in situ*

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Abstract

Adoptive transfer of *in vivo* Concanavalin A (Con A)-activated syngeneic lymphocytes at the tumour site of mice could more effectively curb the MCA-induced tumour growth than injecting Con A, a polyclonal activator for T lymphocytes, in the tumour-bearing hosts. If these effector lymphocytes were depleted of suppressor T cells by treatment with low dose of cyclophosphamide (CY), the inhibition of the tumour growth was better. The cytotoxic ability of these effector cells against ^{51}Cr-labelled tumour-target cells seemed to be doubly effective than the Con A-stimulated cell population as such.

Key words: Polyclonal stimulation, Con A, fibrosarcoma control.

1. Introduction

Several laboratories have shown that stimulation of the reticuloendothelial system of host with certain non-specific agents like BCG[1–4] and other killed microorganisms[5–8] restrict the growth of malignant tumour. T Cell-mediated immune response is considered the effector mechanism in rejection of grafts including neo-antigen-bearing malignant cells[9,10].

Concanavalin A, a polyclonal T-cell stimulator, was found earlier to activate the murine T cells, both *in vitro* and *in vivo* driving the cells all the way to cytotoxic killer cells against targets of H-2 nonidentity and tumour cells[11–17]. We reported some success in curbing the tumour growth as well as the neovascular reactions induced by a growing tumour piece transplanted in the anterior chamber of the eye in syngeneic mice, in the presence of Con A-stimulated lymphocytes[18–19].

These observations obviously raised the question whether polyclonal stimulation of T lymphocytes by Con A would be effective in curbing the growth of solid tumours *in situ*. This has been studied in the present investigation in the following experiments: Polyclonal stimulation of host's own lymphocytes and the adoptive transfer of these isogeneic Con A-activated cells at the tumour site and also after prior removal of suppressor T cells. Subsequently, the efficacy of polyclonally activated cells in killing radiolabelled tumour

target cells before and after the removal of suppressor cells. Since polyclonal stimulator like Con A is supposed to stimulate suppressor subset of T cells also[20-22], depletion of suppressor T cells was achieved with low doses of cyclophosphamide treatment which was shown to be effective in removing suppressor T cells[23-27].

2. Materials and methods

2.1. *Animals*

Breeding nuclei of Swiss albino mice were obtained from the Indian Institute of Chemical Biology (formerly the Indian Institute of Experimental Medicine), Calcutta, and were maintained in our Centre. Male mice of 6–12 weeks of age were used throughout the study.

2.2. *Tumours*

Fibrosarcomas were induced by subcutaneous injection of 2 mg of 3-methylcholanthrene (MCA) (Sigma Chem. Co., St. Louis, USA) in 0.2 ml of Freund's Incomplete Adjuvant (Difco Lab., USA) per animal, anaesthesized with intraperitonial injection of 0.07 mg/g of body wt of thiopentone sodium (May and Baker, Bombay). Incomplete adjuvant was used to prepare suspension of MCA only for its mineral oil base and had no tumour-inducing property. The mean time for appearance of palpable tumour was 15 days.

2.3. *In vivo lymphocyte activation with Con A*

Concanavalin A (type IV, Sigma Chem. Co., USA) dissolved in sterilized distilled water and passed through millipore membrane filter paper (0.45 m porosity) prior to use was injected intravenously at a dose of 50 μg in 0.1 ml per animal.

2.4. *Cell suspension*

Effector cells: Chaudhuri and Chakravarty[28] observed activation of lymphocytes *in vivo* with 50 μg of Con A after 48 h of injection and their method was followed to obtain effector cells for the study. Briefly, the spleen and lymph nodes were aseptically collected and cells were dissociated in PBS (pH 7.0 – 7.2) with the help of stainless steel were mesh and repeated passage through a 27-g needle fitted to a syringe. Erythrocytes in spleen cell suspensions were lysed by exposure to tris-buffered ammonium chloride (0.83%, pH 7.2). Cells were finally suspended in minimum essential medium (MEM: Hi-Media, Bombay) supplemented with 10% goat serum[29].

Tumour-target cells: Cells were collected from the supernatant of the minced fibrosarcoma pieces incubated in 0.25% trypsin in a water bath (37°C) for 45 min with occasional shaking. For radiolabelling, 10^7 cells in MEM were incubated in water bath (37°C) for 1.5 h with 200 μCi of $Na_2^{51}CrO_4$ (sp. act. 130–193 mCi/mg, Bhabha Atomic Research Centre, Bombay), washed thoroughly and resuspended in MEM.

2.5. *Measure of tumour growth and survivality of tumour-bearing host*

Rate of growth of tumours was noted as increase in mean diameter (cm^2) of tumours measured with the help of a slide calliper fitted to a vernier scale at every 7-day interval. The observed values were plotted on the Y axis up to a value of 4 only; the straight line for rate of tumour growth was drawn according to the least-squares fit method, and the slope for the line was calculated. Life span of the tumour-bearing hosts was noted as per cent of living animals at every 7-day interval.

2.6. *Repeated injections of Con A in tumour-bearing mice*

Lymphocytes of tumour-bearing hosts were activated repeatedly by intravenous injections of Con A (50 μg/animal) in two protocols. In one group of animals, only two injections were given: first one, 5 days prior to and the second on the 5th day after MCA injection for tumour induction. In another group of animals, first injection of Con A was given on the day of detection of palpable tumour and subsequent injections, up to 120 days, were given at 10-day intervals. Controls for both the sets were injected with physiological saline.

2.7. *Adoptive transfer of activated lymphocytes at the tumour site*

Three different concentrations of effector spleen and mesenteric lymph node (MLN) cell suspension (5×10^5, 10^6 and 2×10^6 cells in PBS) were injected at 10-day interval, up to 120 days, from the day of detection of palpable tumour. Half of each inoculum was made subcutaneously on either side (180° apart) of a tumour. In controls, non-activated lymphocytes obtained from normal animals were injected following the same schedules. Same protocol was followed for transfering the effector lymphocytes depleted of suppressor T cells excepting that the injections were continued beyond 120 days, as the life span of tumour-bearing mice increased.

2.8. *Depletion of suppressor T cells and subsequent activation of residual cells*

Technique for depletion of suppressor T cells by cyclophosphamide (CY) treatment was adopted chiefly from the works of Ray and Raychoudhuri[24]. Four different doses of CY at lower range, 12.5–100 mg/kg, were tried and the dose capable of effective removal of suppressor T cells was selected on the basis of its enhancement in Con-A responsiveness as noted in Table I.

Cyclophosphamide powder (Sigma Chem. Co., USA) dissolved in sterile distilled water prior to each use was injected intraperitoneally in four different doses (100, 50, 25 and 12.5 mg/kg of body wt/animal) in 0.5 ml of distilled water in different groups of animals. After 48 h, the spleens and mesenteric lymph nodes were collected, made free of fatty tissue and weighed in monopan balance and the per cent reduction in mean wt (Table I) relative to that of control animals (injected with 0.5 ml of distilled water only) was recorded. Con-A responsiveness of the CY-treated animals was noted as per cent increment in mean wt of spleen and mesenteric lymph node 48 h after Con-A injection (done 48 h after CY administration) of these animals. Blastoid lymphocytes in these organs were also recorded.

Table I
Con A responsiveness of lymphocytes from mice treated with different doses of cyclophosphamide (CY)

Dose of CY (mg/kg/animal)	Organs	48 h after CY treatment	48 h after Con-A injection	
		Per cent reduction[a] in mean weight (mg)	Per cent increment[b] in mean weight (mg)	Per cent of blastoid cells[c] ± S. E.
100	Spleen	67	14	18 ± 5.1
	MLN[d]	62	19	28 ± 7.4
50	Spleen	45	2	30 ± 3.7
	MLN	30	−3	36 ± 6.4
25	Spleen	20	31	69 ± 3.0
	MLN	24	37	64 ± 2.4
12.5	Spleen	28	19	44 ± 7.1
	MLN	1	38	56 ± 6.1
Control[e]	Spleen	—	18	41 ± 5.4
	MLN	—	11	48 ± 2.8

[a] Percentage calculated on the basis of reduction in mean (from four animals) weight from that of control animals. In control animals, mean weight of spleen was 82 mg and that of MLN 62 mg.
[b] Percentage calculated on the basis of increment in mean weight of the organs from six animals in reference to that of the control animals.
[c] Background level of blastoid cells in normal animals without any injection was between 6 and 9%.
[d] MLN, mesenteric lymph node.
[e] Injected with 0.5 ml of distilled water (i.p.) instead of CY.

The dose of 25 mg of CY/kg/animal was found to be optimal for depletion of suppressor T-cell activity (Table I) as there was minimum reduction in mean weights of lymphoid organs and maximum gain of the lost weight possibly due to increased blastogenesis in lymphocytes induced by Con A.

2.9. *Cytotoxicity assay*

Aliquots of 10^4-radiolabelled tumour-target cells in 0.25 ml of MEM were mixed with effector lymphocytes in 1 ml of supplemented MEM at different ratios (1:10–1:100) in small glass tubes and incubated in humidified atmosphere at 7.5% CO_2 in air at 37°C for 4.5 h. At the end, radioactivity released in the supernatant (1 ml) of each tube was measured in gamma-ray spectrophotometer (Electronic Corporation of India, Hyderabad) and the percentage of cytotoxicity was calculated as follows:

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

Index for cytotoxicity was calculated as the mean values of triplicates for each point.

3. Results

3.1. *Activation of T lymphocytes in tumour-bearing animals*

Rate of tumour growth in mice, injected intravenously with 50 μg Con A/animal following both the protocols, as indicated in materials and methods, was slower than that of their respective control groups. However, tumour growth in a group of animals injected repeatedly with Con A following the second schedule (Fig. 2a) was slower than in animals injected twice with Con A following the first schedule (Fig. 1a), the respective slope values being 0.7 and 1.11. This slower rate of tumour growth was also reflected in the survivality of the host animals (Figs 1b and 2b).

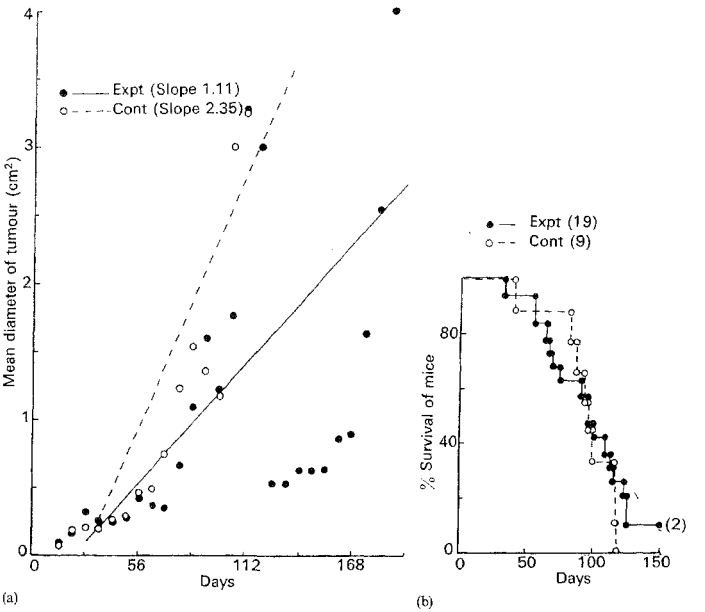

FIG. 1. a. Rate of tumour growth, and b. Survivality of tumour-bearing mice after intravenous injections of Con A. Two injections of Con A on -5 and $+5$ day of MCA injection: (●———) and control, injected with normal saline: (○———).

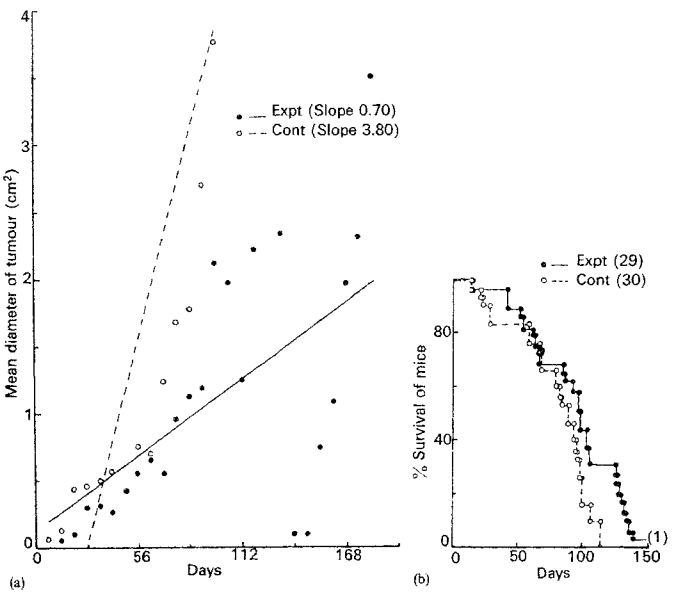

FIG. 2. a. Rate of tumour growth, and b. Survivality of tumour-bearing mice repeatedly injected with Con A after detection of palpable tumour at 10-day intervals up to 120 days: (●————) and control injected with normal saline: (○- - - -).

3.2. *Adoptive transfer of activated lymphocytes at tumour site*

Syngeneic-activated lymphocytes, injected at the tumour site, could curb effectively the growth rate in comparison to the controls where no cells or non-activated cells were injected (Figs 3a, 4a and 5a). The cell dose of 2×10^6/animal was found most effective in suppression of growth rate of tumour (Fig. 5a). In this group, mortality of the host animals was also lower (Fig. 5b); certain percentage of the experimental animals survived until 150 days, 50 days beyond the death of all tumour-bearing control animals.

3.3. *Adoptive transfer of effector cells depleted of suppressor T cells*

A furthermore inhibition in tumour growth was observed when suppressor T cells were removed from the effector cell population injected at the tumour site (Fig. 6). For depletion

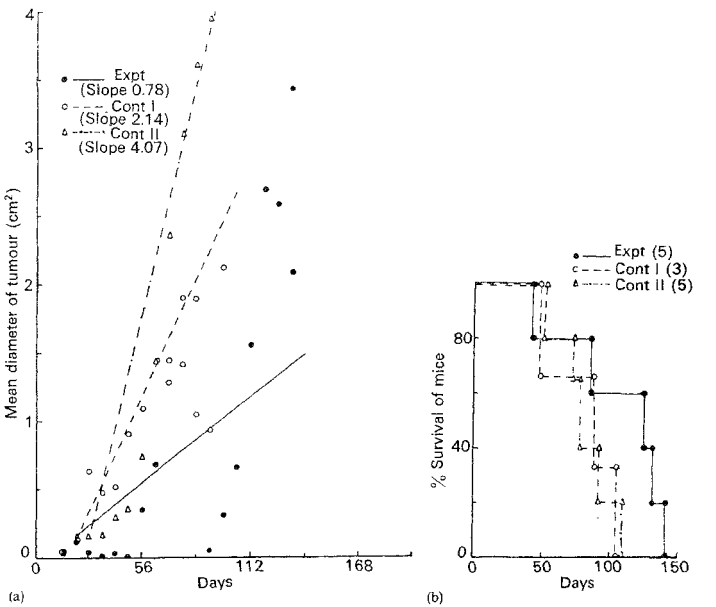

FIG. 3. a. Rate of tumour growth, and b. Survivality of tumour-bearing hosts after injections of Con A-activated lymphocytes at the tumour site at 10-day interval for 12 times. 5×10^5-activated lymphocytes: (●————): control I, normal non-activated lymphocytes: (○– – –) and control II, without any injection: (Δ–·–).

of T cells in an animal, 25 mg CY/kg was administered i.p. 48 h earlier to Con-A injection since this dose of CY was most effective in depleting suppressor T-cell activity (Table I). In Fig. 6, the slopes for tumour growth indicate that higher number of effector cells could restrict the tumour growth better and thus 40% of tumour-bearing animals injected with 2×10^6 cells/animal survived beyond 200 days (Fig. 6b).

3.4. *Cytotoxicity of effector cells after CY treatment*

The functional aspect of the effector lymphocytes raised in animals treated with different doses of cyclophosphamide was tested in ^{51}Cr-release assay. The level of cytotoxicity reasonably increased on pretreatment with two doses of CY, 25 and 12.5 mg/kg of body wt/animal (Table II), over the level obtained with lymphocytes activated without being pretreated with CY (control). Table II indicates that the dose of 25 mg of CY/kg as the

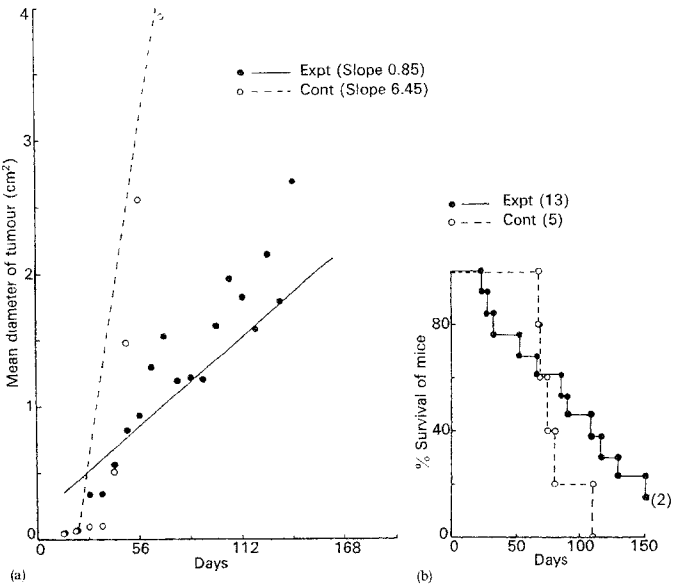

FIG. 4. a. Rate of tumour growth, and b. Survivality of tumour-bearing hosts after injections of 2×10^6-activated and normal lymphocytes at the tumour site at 10-day interval for 12 times. Activated lymphocytes: (●————) and normal lymphocytes: (○- - - -).

most effective in enhancing the response which correlates well with the Con-A responsiveness of these lymphocytes (Table I). However, the level of cytotoxicity did not always correspond with the increment of the ratio of the target to effector cells. Cytotoxic ability of the activated lymphocytes pretreated with 100 mg of CY/kg has not been included in the table which was very poor corresponding to the low blastogenic response of these cells (Table I).

4. Discussion

Adoptive transfer of *in vivo*-activated syngeneic lymphocytes seems to be more effective than stimulating the hosts own immune system directly by intravenous injections of Con A (Figs 3–5 *vs* Figs 1 and 2). These effector lymphocytes have earlier been found to be capable of restricting the growth of tumour piece transplanted in the anterior eye chamber of syngeneic mouse[18,19]. The visual effect of this restriction was revealed from the inhibition

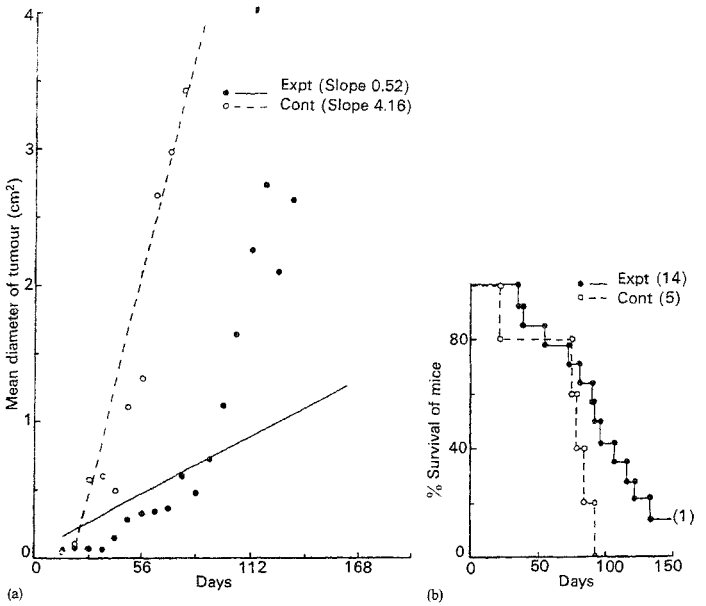

FIG. 5. a. Rate of tumour growth, and b. Survivality of tumour-bearing hosts after injections of 2×10^6-activated and normal lymphocytes at the tumour site at 10-day interval up to 120 days Activated lymphocytes (●————), normal lymphocytes: (○– – –).

in tumour-induced blood vascular proliferation over cornea. A similar effect was observed when the tumour pieces were incubated (*in vitro*) with Con A-activated lymphocytes prior to transplantation; practically, the effector lymphocytes incapaciated such tumour pieces even to incorporate radioactive thymidine *in vitro*[30]. This obviously led to our present attempt of treatment of established tumours *in situ* by adoptive transfer of these polyclonally activated effector cells.

Some workers have suggested passive transfer of sensitized effector lymphocytes to combat neoplastic growth[31,32] but with limited success. So far there are not many reports about using non-specifically raised lymphocytes for adoptive transfer; however, some successes have been reported by transfering T cells expanded in T cell growth factor or in interleukin-2[33–35]. But our present approach of raising effector cells by *in vivo* use of a polyclonal stimulator for T cells possibly makes short the methodical paraphernalia as the earlier works on lectin-mediated activation of effector lymphocytes and the measure

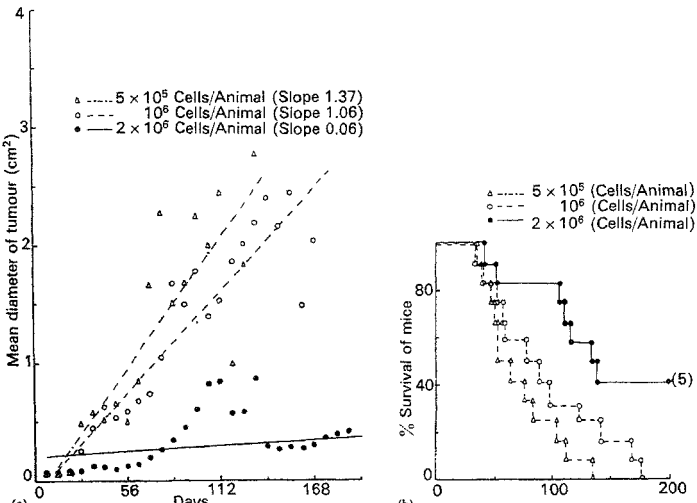

FIG. 6. a. Rate of tumour growth, and b. Survivality of tumour-bearing animals after injections at the tumour site of different quantum of Con A-activated lymphocytes depleted of suppressor T cells at 10-day interval for 16 times. 5×10^5 cells: (Δ–·–·–), 10^6 cells: (○———) and 2×10^6 cells: (●———).

of their lytic function to tumour cells were restricted chiefly in *in vitro* system[11,13,14,36] having less therapeutic orientations.

The second protocol of keeping host's system continuously in active state against neoantigens by repeated Con-A injections (Fig. 2) was obviously better than the first one (Fig. 1). This schedule of repeated stimulation of hosts own system with a polyclonal stimulator obviously made some of the lymphocytes in the hosts reactive to malignant cells which were otherwise non-responsive, possibly due to some suppressor or blocking factor(s)[38–40]. This schedule also raised the possibility of clonal exhaustion of responsive cells. The possibility was tested and no apparent sign of exhaustion of Con A-responsive cells in the animals having such multiple injections of Con A was noted[37]. In the course of this study, a very low titre of anti-Con A antibody was detected in the serum of these animals, which possibly neutralized a part of the Con A injected every time and made this direct stimulation of host's own system repeatedly less effective than repeated transfer of activated lymphocytes at the tumour site of the syngeneic hosts. It may also be possible that the injected Con A bind to certain glycoprotein receptors on the tumour cells and produce a masking effect so that the tumour cells may partially escape destruction by activated lymphocytes of host origin.

Table II
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.	Per cent 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	14 ± 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
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 ± 6.1	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 48 h earlier to Con-A (50 μg/animal, including control) injection.
[b] Effector cells were collected after 48 h of *in vivo* Con-A stimulation.
[c] Spontaneous release varied from 35 to 42% from experiment to experiment.

Con A, a polyclonal T-cell stimulator, is supposed to activate all subsets of T lymphocytes including the suppressor T cells. An attempt was made to find the activity of effector cell population depleted of suppressor T cells with the help of cyclophosphamide pretreatment. Lower doses of CY have been found to be effective in removing the suppressor activity which is reflected in the augmented Con-A responsiveness (Table I) and cytotoxic response (Table II) of the effector lymphocytes. It was found earlier that lower doses of CY selectively remove the suppressor subpopulation of T lymphocytes without causing much toxicity to other cells[23-27]. It seemed that depletion of the suppressor cells indeed increased the effector function of the activated cells in curbing of tumour growth *in situ* (Fig. 6).

Thus, the transfer of syngeneic Con A-activated lymphocytes helped to combat the growth of chemically induced tumours in mice and the response could be augmented by prior removal of the suppressor T cells from effector cell population. Tumour-associated antigens (TAAs) on the malignant cells are likely to make them susceptible to killing by sensitized immunoreactive cells. This recognition and destruction of malignant cells possibly fail in the event of occurrence of tumour. Polyclonal stimulation is likely to stimulate certain clones of T cell to multiply and recognize the TAAs to mount cytolytic reaction. These suggest the feasibility of making the model of polyclonal stimulation of effector lymphocytes against malignancies operative in other experimental animals as well as in man.

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