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Adoptive immunotherapy using human recombinant interleukin-2 activated specific cytotoxic T lymphocytes

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Cytotoxic T Lymphocytes (CTL) generated *in vitro* using mixed lymphocyte tumour culture (MLTC) of primed Balb/c spleen cells (responder) and Dalton's lymphoma ascites (DLA) cells (stimulator) were further expanded in RPMI 1640 + 10% fetal calf serum containing 200 IU/Ml of human rIL-2 at 37°C in 5% CO_2. Activated CTL showed enhanced cytotoxicity against syngeneic DLA cells in 4 hrs ^{51}Cr release assay, when tested on the 7th day of incubation. The antitumour efficacy of the activated CTL *in vivo* was studied using an adoptive immunotherapy model. The growth of DLA tumour transplanted s.c. in the hind leg 7 days prior to the adoptive transfer of 10^7 activated CTL i.p. to tumour bearing mice was significantly suppressed. Furthermore when the tumour bearing recipients were pre-treated with 180 mg/kg of cyclophosphamide 1 hour before cell transfer, effecter cells could produce only a similar effect. The effector cells showed negligible cytotoxicity against NK susceptible cell line YAC-1 lymphoma, Sarcoma 180 and Ehrlich ascites tumour, indicating the specific nature of the effector cells. Pretreatment of the effector cells with anti Lyt-2+ antibody and the complement completely abrogated antitumour activity of the effector cells against DLA cells.

Key Words: CTL, Interleukin-2, DLA.

Cytotoxic T lymphocytes are a subset of antigen induced T lymphocytes with the capacity to recognize, bind and lyse target cells bearing the inducing antigen (4). Usually, the CTL recognizes a target cell bearing a non-self major histocompatibility (MHC) class 1 gene product (K or D in mouse) or an ill defined assemblage of antigens plus a self MHC class 1 gene product. CTL are believed to play an important role in allograft rejection and tumour cell destruction (2). In a weak syngeneic system cytotoxicity mediated by CTL is difficult to detect and often transient. This may be due to the relatively low frequency of effector cells and/or the presence of factors interfering with the lytic mechanism (15). IL-2 has been successfully employed in recent years for long term culture of CTL lines, cloned and uncloned, murine and human derived effector cells produced in MLTC of lymphocytes from sensitized or tumour bearing animals (6). The ability of IL-2 to stimulate cytotoxic T lymphocytes *in vitro,* coupled with successful IL-2 and adoptive cell therapy in several animal tumour models have been reported by many workers (8, 9). In our studies CTL generated *in vitro* exhibited very low cytotoxicity against syngeneic tumour targets (14, 15). The present inves-

tigation attempts to activate *in vitro* generated CTL with human recombinant IL-2 as well as to assess the efficacy of amplified CTL *in vitro* and *in vivo*.

Materials and Methods

Animals and tumours: Pathogen free inbred Balb/c mice were maintained in our animal house. Dalton's lymphoma ascites tumour (DLA) obtained from Chittaranjan Memorial Cancer Research Centre, Calcutta, India was maintained in ascites form by intraperitoneal passages. Balb/c mice 6-8 weeks old were given 10^7 Dalton's lymphoma cells i.p. On the 10th day the tumour bearing mice were sacrificed, the spleen was removed and processed asceptically at 4°C. Sarcoma 180 and Ehrlich's ascites tumour obtained from the Cancer Institute, Bombay and were grown in ascite form in Balb/c mice. The cell line YAC-1 lymphoma obtained from National Tissue Culture Facility, Pune, India was propagated in RPMI-1640 medium plus 10% Fetal Calf Serum (FCS).

In vitro generation and assay of CTL: The detailed methods for *in vitro* generation and assay of CTL have been described previously (14). Briefly, spleen cell suspensions were prepared in RPMI-1640 containing 10% FCS. Erythrocytes were lysed by treatment with Tris Ammonium Chloride buffer pH 7.2. After washing, viable cells were resuspended in complete medium composed of RPMI-1640, Penicillin (100 units/ml), Streptomycin (100 mg/ml), 2mML glutamine, 1mM sodium pyruvate, 5 x 10^{-5}M mercaptoethanol and 10% fetal calf serum (Sigma chemicals). MLTCs were set up in 15ml culture vials, with 1 x 10^7 responder spleen cells and 1 x 10^5 Mitomycin C treated DLA cells in complete medium. After 4 days of incubation at 37°C in a humid atmosphere with 5% CO_2, cells from MLTC were harvested, washed and resuspended in complete medium containing 200 IU/ml of human recombinent IL-2 (Kindly supplied by Dr. Jeffrey Rossio, Lymphokine testing laboratory, NCI Bethesda). Cultures were further incubated at 37°C in 5% CO_2 atmosphere for 7 days, then harvested, washed 3 times and assayed for cytotoxicity against ^{51}Cr labelled DLA targets (14). All tests were done in triplicates. Spontaneous release was determined by incubating target cells with medium alone and total release by incubating in 1 N HCl. The specific lysis was calculated as:

$$\% \text{ specific release} = \frac{\text{Experimental release - spontaneous release}}{\text{Total release - spontaneous release}} \times 100$$

The standard error for triplicate assays was always less than 10%. Spontaneous release was less than 10% of the total release for 4 hrs assay.

Treatment of the effector cells with monoclonal antibody Lyt-2$^+$: About 5 x 10^6 lymphocytes were incubated with appropriate dilution of anti-Lyt-2$^+$ antibody (Sera Lab Ltd.) and fresh normal rabbit serum (1/10 dilution) as complement source for 30 minutes at 37°C. The treated cells were washed twice and assessed for cytotoxicity against radiolabelled DLA target cells as described previously.

Adoptive transfer: Recipient Balb/c mice were inoculated s.c. in the hind leg with 1 x 10^6 DLA cells. On day 7, when the tumour size reached an appreciable diameter, one group of mice were injected with 180 mg/kg wt. of Cyclophosphamide i.p. (Endoxan-ASTA. Khandelwal Laboratories Ltd., Bombay) and 1 hour later 10^7 activited CTL were injected i.p. In another group Cyclophosphamide treatment was omitted and 10^7 activated CTL alone were injected i.p. In the third group of mice only Cyclophosphamide treatment was given. A group of untreated animals served as controls. Tumor volume was calculated as 4/3 x r_1^2 x

Table I - Tumour specificity of *in vitro* generated and rIL-2 activated CTL against different tumour target cells

	% specific lysis (± SD) at Effector: Target ratio of							
	D.L.A.		Ehrlich's ascites		Sarcoma 180		YAC-1	
	10:1	30:1	10:1	30:1	10:1	30:1	10:1	30:1
In vitro generated and IL-2 activated CTL	22.22± 2.4	27.86± 5.06	1.96± 0.40	3.8± 1.16	1.8± 0.66	4.3± 0.75	3.1± 0.60	5.4± 0.82
CTL Treated with anti-Lyt-2^+ antibody and complement	2.2± 0.61	4.3± 0.76	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

N.D.: Not done.

Table II - Results of Winn type neutralization assay of two different syngeneic tumour cells with rIL-2 activated CTL

Tumour	E: T[a]	[b] % of inhibition of tumour take	Tumour volume in cc± SD
DLA[c]	None	0 (0/6)[d]	2.52±0.50
	10:1	100 (6/6)	0.44±0.11**
S 180[c]	None	0 (0/6)	4.9 ±0.26
	10:1	0 (0/6)	4.58±0.43

[a] Effector target cell ratio.
[b] Results were evaluated 3 weeks after tumour transplantation.
[c] 1 x 10^6 tumour cells/mouse s.c.
[d] Numbers in parenthesis, free/total.
** = $P < 0.001$ highly significant.

r_2^2, where r_1 and r_2 are the radii of opposite diameters.

Winn neutralisation assay: IL-2 activated CTL (1 x 10^7 cells) mixed with 1 x 10^6 tumour cells were injected s.c. in the hind leg of syngeneic mice. Tumour size was measured 3 weeks after inoculation of the cell mixtures.

The above experiments were repeated thrice and mean SD of the values was used to calculate significance based on Students t test.

Results

In our earlier studies we have shown that antitumour activity of syngeneic murine CTL was very low with a mean % ^{51}Cr release as 3.85 (14, 15). In the present study the effecter cells were incubated with 200 IU/ml of human rIL-2 for 7 days resulting in a significant enhancement in tumoricidal activity against DLA target cells in 4 hrs. ^{51}Cr release assay (27.86) (Table-I).

Adoptive immunotherapy of transplanted solid tumours by IL-2 activated CTL: As

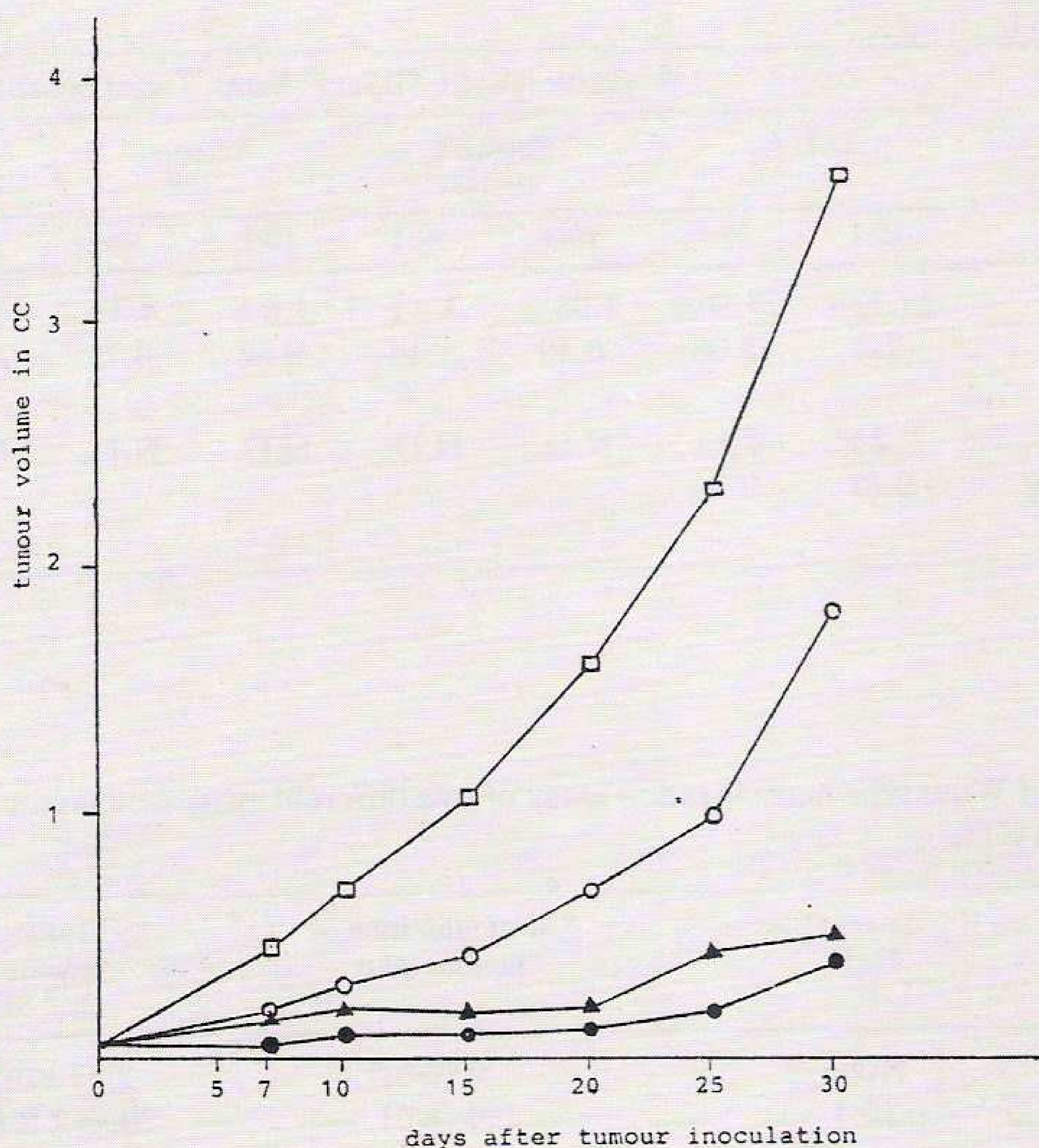

Fig. 1 - Adoptive immunotherapy of DLA tumour bearing Balb/c mice with human recombinant IL-2 activated CTL. Adoptive transfer of activated CTL was done as described in Materials and Methods and tumour volume measured at different intervals of tumour progression. ●, animals treated with cyclophosphamide and activated CTL, ▲, animals treated with CTL alone; ○, animals treated with cyclophosphamide alone, □, untreated control animals.

shown in Fig. 1, 10^7 CTL could suppress the tumour growth significantly in mice transplanted with 1 x 10^6 DLA cells s.c., and treated with 180 mg/kg cyclophosphamide 1 hr prior to the adoptive transfer of activated cells (0.46 ± 0.06). An almost similar effect could be observed in mice treated with 10^7 activated CTL alone. The same dose of cyclophosphamide alone had no suppressive effect on tumour growth. All untreated control tumour bearing mice died within 40 days after tumour inoculation, while 90% of the mice treated with activated CTL survived. The mice who survived were shown to specifically reject DLA cells when they were rechallenged after 60 days.

Specificity of the effector cells: Antitumour effectiveness of the activated CTL was tested against the spectrum of non-specific targets such as YAC-1 lymphoma, Sarcoma 180 and Ehrlich ascites tumour. As indicated in Table I, negligible tumouricidal activity was displayed against these target cells. Non-reactivity with the above targets shows the specific nature of the CTL.

Characterisation of effector cells: Effector cells after activation with rIL-2 were depleted of Lyt-2$^+$ Phenotype cells by treatment with anti-Lyt-2$^+$ monoclonal antibody and complement. As shown in Table 1, treatment of effector cells with anti Lyt-2$^+$ antibody and complement totally abolished the antitumour activity of CTL against DLA cells.

We next examined the target cell specificity of the effector cells *in vivo* by means of the Winn assay. As the data in Table II indicate, the effector CTL could suppress only the growth of DLA tumour cells (Mean tumour volume 0.44) but not Sarcoma-180 (mean tumour volume, 4.58) confirming the specificity of the effector cells.

Discussion

We have previously reported the low tumoricidal capacity of *in vitro* generated syngeneic murine CTL by *in vitro* assays (14, 15). In the present study we have activated the *in vitro* generated CTL using human rIL-2 and tested for the cytotoxic potential *in vitro* as well as *in vivo*. The activated CTL showed enhanced cytotoxicity against DLA cells *in vitro* (Table I) and displayed growth inhibitory activity against DLA cells *in vivo* (Table II). Extending these observations, we further showed that the specific *in vitro* activated CTL were highly effective in suppressing solid tumour growth when adoptively transferred into tumour bearing syngeneic mice (Fig. I).

In addition, no profound enhancement in the tumour reducing property of activated CTL was documented in mice pretreated with cyclophosphamide and given 10^7 CTL i.p. as compared to mice treated with activated CTL alone (Fig. 1). However, several workers have reported cyclophosphamide induced enhancement of therapeutic efficacy of effector cells presumably by the elemination of suppressor cells (11, 12). The cytotoxic potential of the effector cells were tested against a spectrum of non-specific tumour targets including murine NK cell susceptible target YAC-1 lymphoma. No reactivity of the effector cells with these non-specific targets revealed the specific nature of effector cells (Table I). It has been reported that CTL generated against tri nitro phenyl (TNP) modified syngeneic spleen cells displayed negligible cytotoxicity against YAC-1 cells (1). Depletion of effector cells with Lyt-2$^+$ phenotype using specific monoclonal antibody and complement completely abrogated the CTL activity (Table I). Several investigators have shown that the majority of the CTL specific for syngeneic tumours were of Lyt-2$^+$ phenotype (1, 3, 10). In our experiments mice cured by adoptive immunotherapy could also specifically reject DLA cells when they were rechallenged after 60 days of tumour inoculation. The above findings provide ample evidence for the antigen specific T cell nature of the effector cells. It has been reported that leukemic population in mice can be made regressive by treatment with T cells specifically reactive against viral antigens (5). Klarnet et al reported that adoptively transferred T cells in mice resisted for a long time and could reject the challenged tumour (7). Better efficacy of IL-2 expanded tumour infiltrating lymphocytes (TIL) in producing remission of MC 38 colon carcinoma in mice has been reported previously (13).

Summarizing, we have demonstrated that *in vitro* activated antigen specific CTL play a major role in bringing about tumour cell destruction *in vivo* in a specific manner. These studies provide basis for experimentally evaluating the use of CTL in the treatment of various haematopoietic malignancies.

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