Cancer Immunol Immunother (1984) 18: 113–117

Cancer Immunology Immunotherapy

In vitro effects of 4-hydroperoxycyclophosphamide on concanavalin A-induced human suppressor T cells

Abraham Klajman[1], Ilana Drucker[1], Yosef Manor[1], and Shlomo Ben-Efraim[2]

[1] Laboratory for Clinical Immunology and Medical Department, Meir Hospital, Kfar-Saba, Israel
[2] Department of Human Microbiology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv 69978, Israel

Summary. *Treatment in vitro of human peripheral blood lymphocytes (PBL) with ConA induced the generation of suppressor cells which inhibited T cell blastogenic response to ConA and of allogeneic response in the mixed lymphocyte reaction (MLR). Treatment of PBL with 4-hydroperoxycyclophosphamide (4-HPCy) before incubation with ConA markedly decreased the generation of suppressor cells by ConA. The effect of 4-HPCy on generation of suppressor cells was more pronounced in the test of ConA stimulation than in the MLR. Treatment with 4-HPCy had no effect on suppressor cells already induced as shown by incubation of PBL with 4-HPCy after incubation with ConA.*

Introduction

Depletion by cyclophosphamide (CY) of suppressor T cells acting in cell-mediated immune responses in vivo in mice is well documented [1, 5, 8, 11]. More recently, it was reported that treatment of humans with CY increased the acquisition of T-cell-mediated immunity in cancer patients [9] and selectively impaired the generation of non-specific suppressor cells by ConA [2].

The immunoregulatory properties of CY cannot be demonstrated in vitro because its activation requires metabolism by microsomal enzymes in the liver. However, it was shown that a derivative of CY, 4-hydroperoxycyclophosphamide (4-HPCy), is spontaneously converted in aquous solutions to 4-hydrocyclophosphamide, a metabolite that mimics in vitro the in vivo effects of CY [7, 10, 12]. With 4-HPCy it was reported that suppressor T cells participating in the cell-mediated delayed-type hypersensitivity (DTH) response were sensitive to the action of this drug [4, 7], wheras suppressor T cells involved in humoral immune response were resistant to HPCy [4].

It has been reported [10] that ConA-induced T suppression of human B-cell differentiation is abrogated by pretreatment of human T cells with very low concentrations of 4-HPCy, whereas differentiated suppressor T cells are resistant to 4-HPCy. In this study we investigated the effect of 4-HPCy treatment on ConA-induced human suppressor T cells (Ts) in systems based on T-cell activities such as mitogenic stimulation of T cells and mixed lymphocyte reaction (MLR). We show that pretreatment with 4-HPCy inhibited the generation of ConA-induced Ts cells, whereas treatment with 4-HPCy after ConA had no effect on Ts cells already induced.

Offprint requests to: Shlomo Ben-Efraim

Materials and methods

Preparation of lymphocytes. Human peripheral blood was collected from healthy young donors by venipuncture in syringes containing 10 U/ml of preservative-free heparin. Peripheral blood lymphocytes (PBL) were separated on a Ficoll-hypaque gradient [3]. The culture medium used in all experiments was RPMI 1640 (Biolab., Jerusalem, Israel), supplemented with 10 m*M* Hepes buffer (Sigma Chemical Co., St. Louis, USA), 20% inactivated human AB serum, 2 m*M* glutamine, and an antibiotic-antimycotic mixture. All incubations were performed at 37° C in a humidified atmosphere containing 5% CO_2.

ConA induction of suppressor T cells (Ts). ConA-induced Ts cells were prepared as reported elsewhere [13] with a few modifications. Briefly, 3×10^6 PBL/ml in culture medium were incubated with 50 μg/ml ConA (Miles-Yeda Co., Rehovot, Israel), for 48 h. After incubation, the cells were washed in serum-free medium and treated with mitomycin C (MC Sigma, USA), 25 μg/1 $\times 10^6$ cells for 25 min, washed again three times in serum-free medium, and suspended in culture medium in concentrations of 1×10^6 cells/ml. Control cells were prepared in the same way, except that culture medium was added instead of ConA solution.

Treatment of PBL with 4-HPCy. Quantities of 1×10^7/ml PBL were incubated with equal volumes of graded amounts of freshly dissolved 4-HPCy (Asta Werke, Brackwede, W. Germany) in serum-free medium for 60 min. The cells were washed three times in serum-free medium and adjusted to 3×10^6/ml in culture medium. Different concentrations of 4-HPCy (0.3–30 μg/ml) had to be tried in each experiment in view of the lability of the compound in aqueous solution.

Effect of 4-HPCy treatment on ConA-induced Ts

a) Effect on mitogenic response. Freshly prepared PBL (R: responder cells) allogeneic to the PBL used for induction of Ts cells were prepared and adjusted to a concentration of 2×10^6 cells/ml in culture medium. A volume of 0.05 ml responder cells was mixed with 0.05 ml ConA solution (200 μg/ml) and placed in U-shaped microplates (Nunc 1480, Denmark) together with 0.1 ml 2×10^6 cells/ml of one of the following cell suspensions treated with MC: ConA-induced Ts cells (ConA-PBL); control cells not stimulated with ConA (PBL); cells treated first with 4-HPCy and later with ConA (4-HPCy-ConA-PBL), and cells first treated with ConA and

Table 1. Effect of pretreatment with 4-HPCy on ConA-induced T-cell suppression: blastogenic response to ConA[a]

Expt. no.[c]	^{3}H-Thymidine incorporation in PBL supplemented with[b]				
	Untreated PBL cpm ± SD	ConA-treated PBL		4-HPCy-pretreated, ConA-treated PBL	
		cpm ± SD	% Supp[d]	cpm ± SD	% Supp
1	108,984 ± 11,007	73,531 ± 2,807	33	114,180 ± 2,571	− 4
2	102,026 ± 3,026	71,303 ± 5,012	31	83,276 ± 3,360	18
3	85,333 ± 2,659	60,880 ± 3,436	29	93,537 ± 1,592	− 9
4	37,734 ± 4,458	23,563 ± 1,998	38	35,283 ± 5,311	7
5	99,960 ± 14,059	70,995 ± 7,470	29	125,254 ± 18,189	−25
6	57,801 ± 3,360	44,752 ± 5,000	23	58,616 ± 3,231	− 1
7	87,889 ± 7,062	54,074 ± 4,430	39	98,226 ± 20,450	−11
8	49,295 ± 1,400	29,114 ± 2,512	41	22,657 ± 1,504	54
9	60,789 ± 2,400	35,311 ± 2,013	42	49,639 ± 2,218	18
10	153,583 ± 10,582	51,295 ± 3,312	67	118,769 ± 9,414	23
11	51,414 ± 4,846	35,447 ± 1,265	31	61,598 ± 3,065	−19
12	104,340 ± 4,337	35,073 ± 895	66	53,288 ± 9,988	49

[a] All experiments were performed in quadruplicate

[b] Cells added to cultures were inactivated with mitomycin (25 μg/1 × 10^6 cells); untreated PBL (PBL incubated without ConA); ConA-treated PBL (PBL incubated for 48 h (50 μg/3 × 10^6 cells/ml) with ConA for induction of Ts); 4-HPC-pretreated, ConA treated PBL (PBL treated with 4-HPCy for 1 h, washed, subsequently treated with ConA); cpm ± SD: net mean cpm (minus background of cultures incubated without ConA)

[c] Expt. 1 was performed with addition of 4-PHCy 0.3 μg/ml/1 × 10^7 cells/ml; Expts 2 and 3 with 3 μg/ml 4-HPCy; Expts 4 and 9 with 10 μg/ml 4-HPCy; Expts 5−8 with 15 μg/ml 4-HPCy; and Expts 10−12 with 20 μg/ml 4-HPCy

[d] Supp.: % suppression was calculated with reference to cultures supplemented with untreated PBL; the decrease in suppressive activity on ConA-treated PBL due to 4-HPCy pretreatment was statistically significant: $P < 0.005$

Table 2. Effect of pretreatment with 4-HPCy on ConA-induced T-cell suppression of mixed lymphocyte response (MLR)[a]

Expt. no.	^{3}H-Thymidine incorporation in PBL supplemented with[b]					
	Untreated PBL cpm ± SD	ConA-treated PBL		4-HPCy-pretreated, 4-HPCy (μg/ml)	ConA-treated PBL	
		cpm ± SD	% Supp[c]		cpm ± SD	% Supp
1	37,315 ± 1,068	6,051 ± 192	84	6	7,288 ± 330	81
2	52,393 ± 4,255	9,294 ± 689	82	10	19,749 ± 1,964	42
3	57,039 ± 4,896	21,141 ± 1,560	63	15	33,997 ± 2,727	41
4	45,511 ± 2,462	22,297 ± 2,400	51	10	48,862 ± 1,742	− 7
5	35,498 ± 2,462	7,061 ± 1,544	80	15	8,550 ± 824	76
6	69,721 ± 877	14,325 ± 778	80	15	34,870 ± 5,852	50
7	28,104 ± 2,191	3,079 ± 338	89	10	11,519 ± 216	59
8	61,325 ± 4,209	34,243 ± 813	44	20	43,922 ± 3,286	28
9	44,850 ± 896	10,081 ± 1,271	78	20	8,049 ± 618	82
10	53,395 ± 6,174	8,159 ± 816	85	30	16,528 ± 1,494	89

[a] 0.05 ml 2 × 10^6 PBL/ml were cultured with 0.05 ml 2 × 10^6/ml MC-inactivated allogeneic PBL and 0.05 ml 1 × 10^6/ml of MC-inactivated treated cells; after 5 days of incubation 2 μCi tritiated thymidine was added per culture and 18 h later the cultures were harvested; all experiments were performed in quadruplicate and the results are expressed as net mean cpm per well ± SD

[b] See Table 1 for details of treatments of cells

[c] % Supp.: % suppression was calculated with reference to cultures supplemented with untreated PBL; the decrease in suppressive activity due to 4-HPCy pretreatment was statistically significant: $P < 0.05$

later with 4-HPCy (ConA-4-HPCy-PBL). The culture mixtures were incubated for 48 h. A quantity of 2 μCi ^{3}H-thymidine (Nuclear Research Center, Negev, Israel) was added to each well and 18 h later the cultures were terminated by harvesting them on microfiber paper (Whatman GF/C) with an automatic cell harvester (PML Co., Yahud, Israel). The filters were placed in vials containing scintillating fluid and the radioactivity was determined in a beta scintillating counter (Beckman). The results were expressed as net mean cpm per well, i.e., less background cpm in corresponding cultures

incubated without the mitogen. All experiments were performed in quadruplicate.

b) Effect on allogeneic response. MLR was determined as reported elsewhere [6]. Briefly, a volume of 0.05 ml 2×10^6/ml PBL was cultured in the presence of 0.05 ml 2×10^6/ml MC-treated allogeneic PBL. To each well was added either 0.05 ml control PBL (not treated with ConA), ConA-treated PBL, or cells first treated with 4-HPCy and later incubated with ConA. ^{3}H-Thymidine (2 μCi/well) was added on day 5 of incubation. The cells were harvested after an additional incubation period of 18 h. The results were expressed as mean cpm of quadruplicate cultures.

Response of 4-HPCy-treated cells to mitogenic and allogeneic stimulation. PBL cells were treated with various concentrations of 4-HPCy (0.3 μg to 15 μg/1 $\times 10^7$ cells/ml), washed, and adjusted to 1×10^6 cells/ml. The 4-HPCy-treated cells were tested for mitogenic response to PHA (Difco, USA) and ConA (25 μg/ml and 100 μg/ml) in cultures containing equal volumes of 0.1 ml cells and mitogen solution, and for their reactivity in MLR toward allogeneic MC-treated PBL (0.1 ml of 1×10^6 cells/ml).

Statistical analysis. Statistical evaluation was performed by analysis of variance with repeated measures [14]. The differences were considered significant when P was less than 0.05.

Results

Inhibition of ConA-induced T-cell suppression of blastogenic response to ConA by pretreatment with 4-HPCy

PBL cells incubated with ConA consistently depressed the thymidine incorporation in normal PBL stimulated with ConA. The mean suppression was 39%, within a range of 23%–67%. Treatment with 4-HPCy before the induction of suppressor cells by ConA diminished the suppressive effect in 11 of 12 cultures stimulated with ConA. The decrease in activity of ConA-induced suppressor cells due to pretreatment with 4-HPCy was statistically significant (Table 1).

Inhibition of ConA-induced T-cell suppression of MLR by pretreatment with 4-HPCy

Addition of ConA-treated PBL cells to normal PBL cells suppressed their ability to respond to allogeneic stimulation in all 10 cases tested (% suppression from 44% to 84%). Treatment of PBL with 4-HPCy before ConA incubation resulted in a decrease of their suppressive effect in eight of 10 cases (Table 2). This effect was statistically significant ($P < 0.05$).

Inhibition of T-cell mitogenic and allogeneic responses by 4-HPCy

The effect of 4-HPCy on T-cell mitogenic and allogeneic response was examined together with expt 1 of Table 1. PBL were treated with various concentrations of 4-HPCy ranging from 0.3 μg/ml to 15 μg/ml before stimulation with ConA and PHA. A decrease in the blastogenic response to ConA was observed in cultures of cells pretreated with at least 1 μg 4-HPCy/ml, but a decrease in response to PHA was found only in cultures of cells pretreated with 10 μg 4-HPCy/ml (Fig. 1). A decrease in the ability of PBL cells to respond to allogeneic

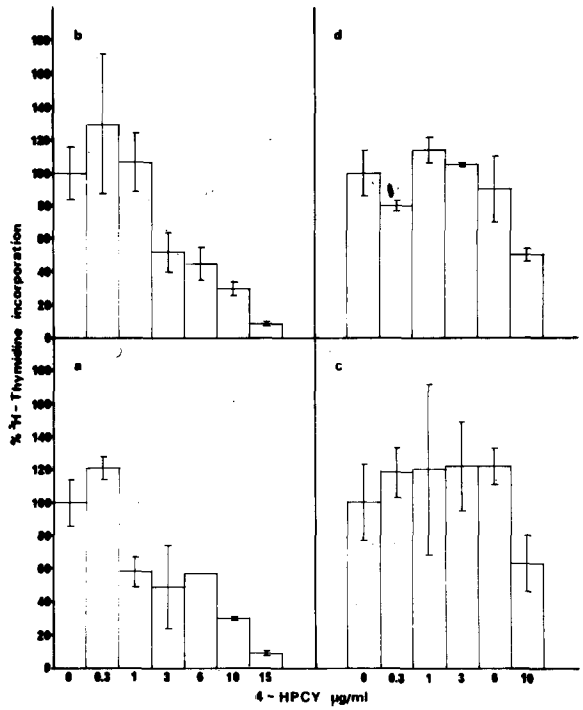

Fig. 1a–d. Response of HPCy-treated PBL to mitogenic stimulation: **a** ConA stimulation with 2.5 μg/1 $\times 10^5$ cells; ^{3}H-thymidine incorporation in 4-HPCy untreated PBL stimulated by ConA was taken as 100% (20,282 ± 2,788 cpm); **b** ConA stimulation with 10 μg/1 $\times 10^5$ cells; ^{3}H-thymidine incorporation in HPCy-untreated cells: 50,705 ± 8,093 cpm (100% value); **c** stimulation with 2.5 μg PHA/1 $\times 10^5$ cells; control value in untreated HPCy cells: 12,796 ± 2,964 (100%); **d** stimulation with 10 μg PHA/1 $\times 10^5$ cells; control value: 64,314 ± 9,264 cpm (100%)

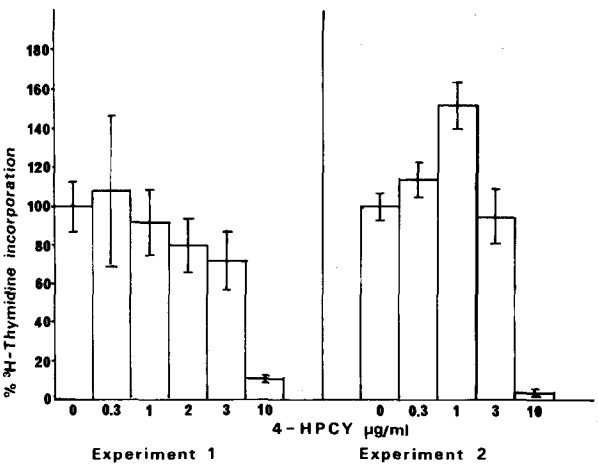

Fig. 2. Response of 4-HPCy treated PBL to allogenic stimulation in MLR: ^{3}H-thymidine incorporation in control cultures of responder cells not treated with 4-HPCy was taken as 100% value: 38,540 ± 5,137 cpm in expt 1 and 70,643 ± 5,037 cpm in expt 2

stimulation in MLR was detected in cultures of cells pretreated with 10 μg 4-HPCy/ml (Fig. 2).

Effect of treatment with 4-HPCy on ConA-induced suppressor cells

The susceptibility to 4-HPCy of ConA-induced suppressor cells was examined in three experiments (Tables 3 and 4).

Table 3. Effect of 4-HPCy treatment after ConA activation on suppression of blastogenic response to ConA[a]

Cells added to ConA-stimulated cultures		ConA stimulation (^{3}H thymidine incorporation)	
		cpm ± SD	%Supp[b]
PBL cultured with media alone		112,262 ± 5,243	
PBL cultured with ConA[c]		69,582 ± 2,703	38
PBL treated with 4-HPCy	0.3 μg[d]	96,777 ± 2,435	14
Only	1.0 μg	90,330 ± 7,913	20
	3.0 μg	92,718 ± 5,975	18
	6.0 μg	124,136 ± 7,553	−10
	10.0 μg	104,550 ± 10,519	7
	15.0 μg	135,107 ± 10,248	−20
PBL treated with 4-HPCy	0.3 μg	52,526 ± 2,376	53
after ConA	1.0 μg	39,525 ± 5,174	65
activation	3.0 μg	61,517 ± 3,126	45
	6.0 μg	62,739 ± 6,667	44
	10.0 μg	73,097 ± 9,679	35
	15.0 μg	84,756 ± 4,383	25

[a] See footnotes to Table 1 for details; the experiment was repeated twice with similar results
[b] %Suppression was calculated with reference to cultures supplemented with PBL cultured with medium alone
[c] 50 μg ConA/3 × 10^6 PBL cells/ml
[d] μg 4-HPCy/1 × 10^7 PBL cells/ml

Table 4. Effect of 4-HPCy on ConA-induced suppression of mitogenic response to ConA[a]

Cells added to ConA-stimulated cultures		ConA stimulation (^{3}H thymidine incorporation)	
		cpm ± SD	%Supp[b]
PBL cultured with media alone		79,314 ± 4,200	
PBL cultured with ConA[c]		38,301 ± 7,954	52
PBL treated with 4-HPCy	0.3 μg[d]	86,258 ± 2,103	− 9
only	10.0 μg	108,560 ± 1,062	−37
	15.0 μg	108,317 ± 12,733	−36
PBL treated with 4-HPCy	3.0 μg	54,018 ± 17,381	32
before ConA	10.0 μg	75,572 ± 14,646	5
activation	15.0 μg	84,828 ± 16,107	− 7
PBL treated with 4-HPCy	0.3 μg	33,023 ± 1,273	58
after ConA	10.0 μg	36,020 ± 4,879	55
activation	15.0 μg	60,490 ± 12,294	24

[a] See footnotes to Table 1 for details
[b] %Suppression was calculated with reference to cultures supplemented with PBL cultured with medium alone
[c] 50 μg ConA/3 × 10^6 PBL cells/ml
[d] μg 4-HPCy/1 × 10^7 PBL cells/ml

Treatment with 4-HPCy after ConA did not affect the activity of ConA-induced suppressor cells on the mitogenic response to ConA. To evaluate the effect of 4-HPCy treatment before or after induction of Ts cells by ConA under similar conditions, an experiment was performed in which the same suspension of PBL was used (Table 4). Pretreatment with 4-HPCy at concentrations of 3 and 10 μg/ml resulted in a marked decrease of ConA-induced suppression, whereas treatment with 4-HPCy after ConA induction of Ts cells did not affect their suppressive effect.

Discussion

We report here that pretreatment of human PBL with 4-HPCy inhibited the generation of suppressor T cells by ConA. This was shown by testing the suppressive effect of MC-inactivated ConA-incubated PBL on cultures submitted to ConA mitogenic stimulation and to allogeneic stimulation in MLR. The effect of pretreatment with 4-HPCy on the ConA-induced generation of suppressor cells was apparently more marked in tests of mitogenic stimulation than in MLR tests. This can be attributed to differences in susceptibility to 4-HPCy between suppressor cells that regulate a mitogenic response and suppressor cells that regulate the allogeneic response or to differences between the two systems used (ConA stimulation vs MLR). Thus, in an MLR system an additional population of lymphocytes is present, which not only acts as an antigenic stimulus, but which may also provide extra suppressor cells to the culture. It should be mentioned that a difference in susceptibility to 4-HPCy of suppressor T cells was reported with murine lymphocytes [4]: Ts cells involved in the DTH reaction were sensitive to 4-HPCy, whereas Ts cells acting in the humoral antibody response were resistant.

Treatment with 4-HPCy after generation of Ts cells by ConA did not affect their suppressive activity on the blastogenic response to ConA. This finding indicates that mature suppressor T cells (in contrast to precursors of Con-induced suppressor T cells) are relatively resistant to 4-HPCy and again shows the heterogeneity of T-cell populations with regard to their sensitivity to 4-HPCy.

The possibility that the effect of pretreatment with 4-HPCy on induction of Ts cells by ConA might be due to overall inhibition of blast transformation by the drug should be considered. We found in this respect that treatment with 4-HPCy inhibited the induction of blast transformation by ConA and PHA and the activity of responder cells in MLR. However, it seems that the inhibitory effect of 4-HPCy on the induction of Ts cells by ConA cannot be solely attributed to overall inhibition of lymphocyte activation, for the following reasons: in concomitant experiments the quantity of 4-HPCy required for inhibition of ConA induction of blast transformation by mitogens (Fig. 1) and for inhibition of MLR (Fig. 2) was smaller than that required for inhibition of Con A induction of Ts cells (Table 1, Fig. 1); and treatment of Ts cells already induced by ConA with 4-HPCy up to 10 μg/ml did not affect their suppressive activity on mitogenic stimulation.

Recently [10], examinations of the in vitro sensitivity of functional human T-cell subsets to 4-HPCy in a polyclonal B-cell differentiation assay and in the generation of Ts for effector B-cell functions have revealed that ConA-induced T-cell suppression of B-cell differentiation and function was completely abolished by pretreatment with 4-HPCy. The abolition by 4-HPCy of ConA induction of Ts cells was observed at low concentrations of 4-HPCy which do not affect blast transformation and DNA cross-linking [10]. The same authors [10] also found that ConA-induced Ts cells are resistant to 4-HPCy. Our results are in agreement with their findings, insofar as they show that pretreatment with 4-HPCy affects ConA induction of suppressor cells and does not affect the activity of Ts cells already induced in test systems based on evaluation of T-cell function rather than evaluation of B-cell function.

Acknowledgements. This work was supported in part by the Moise and Frida Eskanasy Institute for Cancer Research, Tel-Aviv University, Tel-Aviv, Israel.

References

1. Askenase PW, Haysten BJ, Gershon RK (1975) Augmentation of delayed type hypersensitivity by doses of cyclophosphamide which do not affect antibody responses. J Exp Med 141: 697
2. Berd D, Danna V, Maguire H, Mastrangelo M (1982) Selective impairment of suppressor T cell function after administration of cyclophosphamide. Proc Am Assoc Cancer Res 23: 279
3. Boyum A (1968) Separation of leukocytes from blood and bone marrow. Scand J Clin Lab Invest Suppl 21: 97
4. Diamantstein T, Klos M, Hahn H, Kaufmann SHE (1981) Direct in vitro evidence for different susceptibilities to 4-hydroperoxycyclophosphamide of antigen-primed T cells regulating humoral and cell mediated immune responses to sheep erythrocytes. J Immunol 126: 1717
5. Gill HK, Liew FY (1978) Regulation of delayed type hypersensitivity. Eur J Immunol 8: 172
6. Hallgren HM, Yunis EJ (1977) Suppressor lymphocytes in young and aged humans. J Immunol 118: 2004
7. Kaufmann SHE, Hahn H, Diamanstein T (1980) Relative susceptibility of T cell subsets involved in delayed-type hypersensitivity to sheep red blood cells to the in vitro action of 4-hydroperoxycyclophosphamide. J Immunol 125: 1104
8. L'Age-Stehr J, Diamanstein T (1978) Induction of autoreactive T lymphocytes and their suppressor cells by cyclophosphamide. Nature 271: 663
9. Maguire H, Berd D, Engstrom P, Manstrangelo M (1981) Cyclophosphamide increases the acquisition of T cell immunity in patients with melanoma and colo-rectal cancer. Proc Am Assoc Cancer Res 22: 279
10. Ozer HJ, Cowens W, Colvin M, Blumenson A, Sheedy D (1982) In vitro effect of 4-hydroperoxycyclophosphamide on human immunoregulatory T subset function. J Exp Med 155: 276
11. Shand FL (1979) Review/Commentary. The immunopharmacology of cyclophosphamide. J Immunopharmacol 1: 165
12. Shand FL, Howard JG (1979) Induction in vitro of reversible immunosuppression and inhibition of B cell receptor regeneration by defined metabolites of cyclophosphamide. Eur J Immunol 9: 17
13. Shou L, Schwartz SA, Good RA (1976) Suppressor cell activity after concanavalin A treatment of lymphocytes from normal donors. J Exp Med 143: 1100
14. Winer BJ (1971) In: Winer BJ (ed) Statistical principles in experimental design, 2nd edn. McGraw-Hill, London New York

Received March 20, 1984/Accepted July 2, 1984