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In vitro selective effect of melphalan on human T-cell populations

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Summary. *In vitro treatment with 2 μg/2 × 10^6 cells melphalan (L-PAM: L-phenylalanine mustard) significantly decreased the total number of T lymphocytes from peripheral blood (PBL) of healthy human donors and of the OKT_4 population (precursor suppressor/helper/inducer) T cells as defined by monoclonal antibodies OKT_3 and OKT_4, respectively. No changes in the OKT_8^+ lymphocyte population (cytotoxic/mature suppressor cells) were observed following the same treatment. Preincubation of PBL with L-PAM at concentrations that do not affect the rate of DNA synthesis in PHA-stimulated lymphocytes inhibited the generation of T suppressor lymphocytes by ConA, as shown by their effect on PHA stimulation. Treatment of allogeneic PBL with L-PAM had no effect on mature suppressor T cells already induced by Con A, as shown by incubation of PBL with L-PAM after incubation with ConA.*

Introduction

Successful chemotherapy of established tumors has been associated in certain experimental systems with immunomodulation of host's antitumor response by the drug [1, 13]. The cooperation between the effect of the drug and the antitumor immune response was explained either by a change in the kinetics of tumor development induced by chemotherapy [4] or by the selective elimination of various suppressor cell populations by chemotherapeutic agents. In this respect, it was shown that cyclophosphamide (CY) acted selectively against suppressor T cells involved in cell-mediated immune responses in mice [7, 9, 14], increased T-cell-mediated immunity in cancer patients [11], and inhibited the generation of nonspecific human suppressor cells by ConA [8, 15].

L-PAM, like CY, is an alkylating drug and widely used in human neoplasia, especially in multiple myeloma [3, 5, 10, 12]. We have recently shown [1] that treatment with low, nonimmunosuppressive doses of L-PAM cured BALB/c mice of large MOPC-315 plasmacytoma tumors and induced long-lasting antitumor immune response. A similar effect was previously described when CY was used against MOPC-315 plasmacytoma [13]. In view of the similarities between the chemotherapeutic effects of CY and L-PAM, both of which are alkylating agents, it was of interest to determine the effect of L-PAM on T-cell populations. It should also be mentioned that L-PAM, unlike CY, exerts its cytotoxic effect in vitro or in vivo directly, i.e., not by metabolic intermediates. This property allows an accurate examination of the effect of the drug on lymphoid cells in vitro.

In this study we investigated the effect of L-PAM in vitro on human T-cell populations, with especial emphasis on its influence on ConA-induced suppressor T cells.

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Materials and methods

Preparation of lymphocytes. Peripheral blood lymphocytes (PBL) were obtained from healthy donors by Ficoll-Hypaque centrifugation of heparinized blood [2]. In all experiments the culture medium used was RPMI 1640 (Biolab., Jerusalem, Israel) supplemented with 10% pooled human serum and antibiotic mixture. All incubations were performed at 37° C in a humidified atmosphere containing 5% CO_2.

Treatment of PBL with L-PAM. L-PAM powder (Burroughs-Wellcome Co., Research Triangle Park, NC, USA) was used. A concentrated solution of 10 mg/ml was prepared immediately before use in a solvent mixture composed of alcohol: acid (5:1 of 95% ethyl alcohol: 2 *N* HCl). Further dilutions were made in serum-free medium RPMI 1640. For treatment with L-PAM, 2 × 10^6/ml PBL were incubated for 1 h with various concentrations of L-PAM, washed in serum-free medium, counted in 0.1% trypan blue for viabilitity, and adjusted to 10 × 10^6 ml PBL. For T-cell subpopulations, cells were treated with 2 μg L-PAM/2 × 10^6 cells; for PHA stimulations, concentrations of 1, 3, 10, and 20 μg L-PAM/2 × 10^6 PBL were used, and for ConA-induced suppression, cells were treated with 0.3 and 1 μg L-PAM/2 × 10^6 PBL. For all assays, control cells were also preincubated in the same manner as L-PAM-treated cells, with the solvent mixture appropriately diluted in RPMI medium in the absence of the drug (diluent control).

Effect of L-PAM treatment

a) Effect on T cell subpopulations. Phenotypic analysis of T-cell populations was performed by indirect immunofluorescence with the monoclonal anti-OKT_3 antibodies (total peripheral T lymphocytes), anti-OKT_4 antibodies (precursors of suppressor T cells/inducer/helper T-lymphocytes), and anti-OKT_8 antibodies (mature suppressor/cytotoxic T lymphocytes). In brief, 10^6 lymphocytes previously treated with L-PAM or not were incubated with 5 μl OKT_4, OKT_3, or OKT_8 antibody (Ortho-Diagnostic System Inc., NJ, USA) for 30 min at 4° C. After washing, 0.1 ml of 1:10 diluted fluorescent conjugated

goat antimouse IgG (Tago Inc., Immunodiag. Reagents, Burlingame, USA) was added to the cell pellet, thoroughly mixed, and incubated for another 30 min on ice. After two washings in PBS the cells were resuspended in mounting medium (phosphate-buffered saline containing 30% glycerol). Three hundred cells were examined for fluorescent staining with a Zeiss microscope with epi-illumination.

b) Effect on mitogen stimulation. Cultures were prepared by standard techniques. L-PAM-treated and -untreated cells were incubated for 4 days in the presence or absence of 2 μg PHA/1 × 10^6 cells in flat microplates (Falcon, Becton, Dickinson and Co., Calif, USA) at a concentration of 2 × 10^5 cells/well. On day 4, cultures were pulsed for 5 h with 1 μCi ^{3}H Tdr (Nuclear Research Center, Negev, Israel), harvested with an automatic cell harvester (PML Co., Yahud, Israel), and assayed for radioactivity by liquid scintillation in a beta-scintillation counter (Beckman). All experiments were performed in quadruplicate.

c) Effect on induction of suppressor T cells by ConA. Induction of suppressor T cells (Ts) by ConA in L-PAM-treated and -untreated lymphocyte cultures was tested. Briefly, 2 × 10^6 PBL/ml in culture medium were incubated with 20 μg ConA/ml (Miles-Yeda Co., Rehovot, Israel) for 48 h. Cells in the absence of ConA were incubated in the same way. After incubation, ConA-treated and -untreated cells were washed in serum-free medium and treated for 30 min with mitomycin C (Sigma, St. Louis, USA), 40 μg/2 × 10^6 cells. After repeated washings, cells were adjusted in culture medium to 10 × 10^6 lymphocytes/ml and assayed for their suppressive activity on mitogen stimulation of allogenic normal fresh lymphocyte cultures by PHA.

Effect of Ts on mitogenic stimulation. Freshly prepared allogeneic PBL were used as responding cells (R) to measure the suppressive effect of ConA-induced Ts cells. Equal volumes (0.05 ml) of fresh and mitomycin C-treated lymphocytes, adjusted to the same concentration of 10 × 10^6 cells/ml, were mixed in culture medium in the presence of PHA (2 μg/ml), placed in microplates as mentioned before, and cultured for 4 days. The following cell suspensions treated with mitomycin C were mixed with allogeneic R cells: ConA-induced Ts; control cells incubated without ConA; cells treated with L-PAM and subsequently incubated with ConA; cells treated with L-PAM and subsequently incubated without ConA; cells incubated with ConA and subsequently with L-PAM; cells incubated without ConA and subsequently treated with L-PAM; control cells incubated with ConA subsequently treated with L-PAM diluent control (in the absence of L-PAM); cells incubated without ConA and subsequently treated with diluent only.

Results and statistical analysis

For mitogen stimulation and for suppression, data are presented as 10^3 counts per minute (cpm) ± SE. The percentage of suppression was calculated as follows:

$$1 \frac{\text{cpm} \times 10^3 \text{ in presence of ConA-treated cells}}{\text{cpm} \times 10^3 \text{ in presence of ConA-un treated cells}} \times 100.$$

For statistical evalution, Student's paired matched *t*-test was used. The differences were considered significant when *P* was less than 0.05.

Results

Decreased percentage of OKT_3 and OKT_4 lymphocytes after treatment with L-PAM

Incubation of PBL with L-PAM 2–3 μg/2 × 10^6 cells significantly decreased both the percentage of total T lymphocytes and the percentage of OKT_4 cells (precursors of suppressor T cells/inducer/helper cells) as defined, respectively, by monoclonal antibodies OKT_3 and OKT_4. The effect was selectively directed against OKT_4 lymphocytes, which decreased by 27%–50% in all five cases tested, whereas the percentage of OKT_8 lymphocytes (mature suppressor/cytotoxic lymphocytes) remained unchanged after incubation with the same concentration of L-PAM (Table 1).

Inhibition of T-cell mitogenic response by L-PAM

PBL were treated with four different concentrations of L-PAM, ranging from 1 to 20 μg/2 × 10^6 cells, before stimulation with PHA (Table 2). The percent viability as tested by trypan blue dye exclusion was not affected by treatment of cells with various concentrations of L-PAM: the percentage of trypan-blue-stained cells was not higher than 12. A marked decrease in ^{3}H-dr uptake in PHA-stimulated cultures was observed when cells were preincubated with 3 μg L-PAM/2 × 10^6 cells, and ^{3}H-Tdr uptake was almost completely inhibited by an L-PAM concentration of 20 μg/2 × 10^6 cells (Table 2). Pretreatment of cells with L-PAM 1 μg/2 × 10^6 cells did not appreciably affect the rate of stimulation by PHA.

Inhibition of ConA-induced T-cell suppression of PHA stimulation by pretreatment with L-PAM

Addition of PBL incubated with ConA consistently depressed thymidine incorporation in normal allogeneic PBL stimulated with PHA (Table 3). The mean suppression was 33% and the

Table 1. In vitro effect of L-PAM on T-cell subpopulations of human lymphocytes

Expt no.[a]	Percent of cells stained by monoclonal antibody[b]					
	OKT_3		OKT_4		OKT_8	
	L-PAM treated[c]	Untreated[d]	L-PAM treated	Untreated	L-PAM treated	Untreated
1	59	80	27	40	20	27
2	51	70	28	38	20	23
3	50	67	27	43	24	22
4	65	70	29	47	22	21
5	44	66	23	39	23	20
Mean ± SE	53 ± 3.6	70 ± 2.5	27 ± 1.0	41 ± 1.6	24 ± 0.8	22 ± 1.2
P[e]	< 0.01		< 0.001		NS	

[a] Each experiment performed with PBL from an individual donor
[b] Percent of stained cells calculated by counting 300 cells
[c] L-PAM-treated cells (2 μg/2 × 10^6 cells); PBL incubated for 1 h at 37° C with L-PAM, thoroughly washed, and stained with monoclonal antibody
[d] Untreated cells: PBL incubated with diluent without L-PAM
[e] *P* calculated for comparison between L-PAM treated and corresponding untreated controls

Table 2. In vitro effect of L-PAM on PHA stimulation of human lymphocytes

Expt. no.[a]	PHA stimulation[b] (cpm × 10^3 ± SE) following treatment with L-PAM (μg/2 × 10^6 cells)[c]				
	–	1	3	10	20
1	23.2±2.1	18.7± 0.9	10.4±1.2	2.8±0.3	1.4±0.1
2	13.5±0.9	12.5± 1.3	4.7±0.4	1.0±0.1	0.7±0.05
3	59.5±4.8	46.3± 3.5	19.8±2.0	0.8±0.06	0.2±0.01
4	45.1±4.2	55.1± 4.1	34.7±2.6	3.2±0.4	0.1±0.01
Mean SE	35.1±10.2	33.1±10.3	17.4±6.5	1.9±0.6	0.6±0.2

[a] Each experiment performed with PBL from one donor
[b] 4-day cultures in the presence of PHA 2 μg/1 × 10^6 cells; means of four parallel samples
[c] 1-h incubation at 37° C and washing before culturing in presence of PHA

Table 3. Effect of pretreatment with melaphalan on ConA-induced T-cell suppression of blastogenic response to PHA

Expt[a]	^{3}H-thymidine incorporation in PBL supplemented with[b]					
	Untreated PBL (cpm)	ConA-treated PBL[c] (cpm)	L-PAM-pretreated/ConA-treated PBL			
			%Supp[d]	L-PAM (μg)[e]	cpm	%Supp
1	47,323	32,708	31	1.0	43,122	9
2	25,600	17,300	33	1.0	29,305	−14
				0.3	24,481	4
3	25,400	15,348	40	1.0	23,363	8
				0.3	21,597	15
4	105,600	67,675	36	1.0	107,712	− 2
5	53,800	40,581	25	1.0	48,753	9
				0.3	42,802	21
6	33,725	24,300	28	1.0	28,755	15
				0.3	29,445	13

[a] Each experiment performed with PBL from one donor
[b] PHA (2μg/1 × 10^6 cells was added at the beginning of the incubation period of 4 days; ^{3}H-thymidine (1 μCi) was added for the last 5 h of incubation; control of cultures without PHA (containing untreated PBL, ConA-treated PBL, or L-PAM-pretreated cells) incorporated ^{3}H-thymidine at the rate of 0.3 × 10^3 to 1.6 × 10^3 cpm); each experiment was performed in quadruplicate and SE did not exceed ± 10 in any of the experiments between the four parallel samples; cultures of 0.2 ml contained mixtures of equal volumes of mitomycin-treated preincubated cells (5 × 10^5/ml) and of fresh responding allogeneic cells (5 × 10^5/ml)
[c] 2 × 10^6 PBL/ml were incubated with 20 μg ConA/ml for 48 h; cells incubated without ConA (untreated PBL) were used as controls
[d] Supp.: %suppression was calculated in reference to cultures supplemented with untreated PBL; the decrease in suppressive activity of ConA-treated PBL due to L-PAM pretreatment was statistically significant: $P < 0.05$
[e] Melphalan (L-PAM) pretreatment: 1 or 0.3 μg L-PAM/2 × 10^6 PBL was added; the cells were incubated with L-PAM for 1 h at 37° C, and thoroughly washed before with ConA

range, 26%–50%. Treatment with L-PAM, 0.3 μg or 1 μg/2 × 10^6 cells, before incubation of PBL with ConA prevented or markedly diminished induction of Ts cells by ConA (Table 3). The highly cytotoxic effect of L-PAM on ConA-inducible suppressor cells was observed in all cultures in

Table 4. Effect of melphalan treatment after ConA activation on suppression of blastogenic response to PHA[a]

Cells added to PHA stimulated cultures	PHA stimulation (^{3}H-thymidine incorporation)			
	Experiment 1		Experiment 2	
	cpm	% Supp	cpm	% Supp
PBL cultured with medium alone	23,346		26,886	
PBL cultured with ConA[b]	5,199	78	4,538	83
PBL treated with L-PAM after ConA activation	4,706	80	2,284	92

[a] See Table 3 for details
[b] Cells incubated with the L-PAM diluent only, under the same conditions as incubation with L-PAM
[c] Melphalan (L-PAM): 1 μg/2 × 10^6 cells

concentrations of the drug of 0.3 and 1 μg/2 × 10^6 cells (Table 3). As can be seen from Table 2, the concentration of 1 μg/2 × 10^6 cells was harmless to DNA synthesis of nonsuppressed PHA stimulated cultures.

Effect of L-PAM treatment on ConA-induced suppressor cells

The susceptibility of previously induced ConA suppressor cells to L-PAM was examined in two cases at the same concentration of L-PAM as before induction of suppression (Tables 3 and 4). Treatment with L-PAM after ConA did not affect the activity of ConA-induced suppressor cells on the mitogenic response to PHA (Table 4).

Discussion

We herewith report that exposure in vitro of human peripheral blood lymphocytes to L-PAM inhibited the expression of specific T-cell antigens as tested by monoclonal antibodies. A significant decrease in OKT_3 T cell surface markers (total, peripheral human T lymphocytes) and in the OKT_4 population was found. In contrast to this, the subset of cytotoxic/mature Ts cells, as defined by the monoclonal antibody OKT_8, remained unchanged after exposure to L-PAM under conditions in which the OKT_4 population was affected. This selective susceptibility of L-PAM to specific T-cell subsets is also reflected in functional tests. The selective susceptibility of specific T-cell subsets to different amounts of L-PAM was clearly evident in the inhibition of ConA-inducible Ts cells. The concentration of L-PAM required to inhibit the generation of ConA-induced Ts cells was lower than that required to inhibit the mitogenic response to PHA (Tables 2 and 3). Similarly to exposure to 4-HP (the active derivative of the alkylating agent CY), in vitro exposure of lymphocytes to L-PAM in concentrations not harmful to blast transformation and DNA synthesis seems to prevent the maturation of T-presuppressor cells to Ts-effector cells [8, 15]. In contrast to this, ConA-induced mature Ts cells were not further susceptible to subsequent treatment with L-PAM, and the suppressor effect was similar to that of cells treated in the absence of the drug (Table 4).

Differences in the susceptibility of suppressor T cells to the alkylating agent CY were reported on murine lymphocytes [6]

and on human lymphocytes, including Ts-cell effects on both B [15] and T lymphocyte functions [8]. Recent studies have suggested functional heterogeneity within the OKT_4+ subset [15–17]. It was observed that ConA-inducible Ts cells may be generated within the OKT_4^+/OKT_8^- subset [15]. We report here for the first time on differences in the susceptibility of OKT_4 and OKT_8 lymphocytes to treatment with L-PAM in vitro and on differences in the susceptibility of ConA-inducible Ts cells to this drug. Our results are in agreement with the findings reported on the alkylating agent CY [15], which suggest that ConA-inducible presuppressor T cells within the OKT_4^+ subset are more susceptible to the cytotoxic effect of the drug than mature Ts cells. Although it might be that L-PAM affects helper cells within the OKT_4 subset, our results nevertheless suggest that, at least at a low concentration, there is a selective effect in vitro of L-PAM on specific human Ts cells.

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