



Effects of immunoregulatory drugs of human peripheral blood T lymphocytes function *in vitro*

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BACKGROUND: *The purpose of the study was to evaluate the mode of action of different immunoregulatory drugs in lymphocyte proliferation and activation.*

METHODS: *The drugs studied were prednisolone (PRED), cyclosporin A (CsA), the recombination of PRED and CsA, L-asparaginase and cytosine-arabinese (ara-C). Peripheral blood lymphocytes from normal blood donors were stimulated by phytohemagglutinin (PHA). Lymphocytes proliferation and activation were determined by tritiated thymidine ([³H] TdR) incorporation, secretion of interleukin-2, level of soluble interleukin-2 receptors in the supernatant of the culture medium, and immunophenotyping analysis of T lymphocyte subsets.*

RESULTS: *Among PRED, CsA and their combination, the strongest inhibition of cell proliferation was induced by PRED while L-asparaginase and ara-C inhibited PHA-stimulated T cells proliferation in concentration and time dependent manner. Among PRED, CsA and their combination, CsA induced the greatest inhibition of IL-2 production. All the immunoregulatory drugs inhibited lymphocyte proliferation and expression of activation antigens.*

CONCLUSION: *The immunoregulatory drugs inhibit both lymphocyte proliferation and activation, but in a different way.*

KEY WORDS: *T Lymphocytes; Prednisolone; Cyclosporine; Cytarabine; Asparaginase*
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INTRODUCTION

T cell activation plays a crucial role in the initiation and regulation of the immune response (1). Activation of T lymphocytes is a complex process. It is characterized by secretion of the lymphokine interleukin-2 (IL-2) and by expression of the membrane receptor for IL-2 on the T cell. Stimulation of this receptor by the secreted IL-2 leads in turn to T cell proliferation (2). T cell proliferation is estimated by measuring H³-thymidine incorporation. Inhibition of T cell activation, therefore, provides a powerful approach for immunosuppressive treatment (3). Evaluation of specific biochemical, molecular and immunologic events associated with T cell activation should become a routine analysis in the clinical laboratory in order to identify defects that result in

impaired cellular immune effector functions (4-6). Quantifying T cell activation may become useful in monitoring the effect of immunomodulating therapies. The aim of this study was to investigate the effect of immunomodulating drugs on the function of resting and activated lymphocytes from normal blood donors.

MATERIALS AND METHODS

Lymphocyte preparation

Peripheral blood monocytes were separated from the heparinized peripheral blood of healthy blood donors by Ficoll-Hypaque (Sigma) density gradient centrifugation. These cells were washed three times with RPMI 1640 medium (Seromed Biochrom KG) and were suspended in the same medium. Cell viability, determined by trypan blue dye exclusion, was more than 96%. CD2⁺ T cells were separated by positive isolation and detachment using CELLlection™ Dynabeads (Dyna, Biotech), which recognizes the CD2 transmembrane glycoprotein. Positively isolated CD2⁺ cells showed >95% purity and viability. These cells are fully responsive to mitogen stimulation.

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Lymphocyte cultures

CD2⁺ cells were cultured (10⁶ cells/well) in sterile 48-well flat bottomed microculture plates (Costar) in 1 ml of RPMI 1640 supplemented with L-glutamine, 10% dialysed fetal calf serum (FCS), 100 µg/ml streptomycin, 100 U/ml penicillin and amphotericin 250 ng/ml (complete medium). All the cultures were set up in triplicate and incubated in a 5% CO₂ air humidified incubator. CD2⁺ cells were stimulated with PHA for 48 hours. The concentration for purified PHA was 5 µg/ml/10⁶ cells (PHA, Biochrom KG). Resting and activated T cells were treated either with CsA, or PRED, or with the combination of CsA and PRED, and with either L-asparaginase, or ara-C. Different concentrations of the above drugs were used (Table 1).

Table 1. Different concentrations of immunoregulatory drugs

Immunoregulatory drugs	Concentrations			
	1	2	3	4
Prednisolone (µg/ ml/10 ⁶ cells)	10	25	40	50
Cyclosporin A (ng/ml/10 ⁶ cells)	100	200	300	400
Prednisolone (µg/ml/10 ⁶ cells)+ Cyclosporin A (ng/ml/10 ⁶ cells)	10+100	25+200	40+300	50+400
Ara-C (µg/ ml/10 ⁶ cells)	4	6	8	10
L-asparaginase U/ml/10 ⁶ cells	4	6	8	10

The proliferative response of cultured T cells was assessed by 3H-thymidine (3H-TdR) incorporation. Cultures were pulsed with 3H-TdR (27 Ci/mmol, Amersham) in a final concentration of 0.22 µCi/ml cultures for the last eight hours of culture. After the cells were harvested the radioactivity was measured by liquid scintillation spectroscopy. Results were expressed as mean cpm/min of triplicate experiments.

T cell activation and proliferation was estimated by the measurements of T cell surface receptor expression such as CD2, CD3, CD4, CD8 and CD25. We used fluorescence monoclonal antibodies (DAKO). The percentage of the above cell surface receptor expression was measured by immunofluorescence analysis. T cell activation was also assessed by quantification of IL-2 and sIL-2R production in the culture supernatants. To determine the production of IL-2 and sIL-2R, the supernatants from activated and resting cells, treated with immunoregulatory drugs, were collected and stored at -30°C. IL-2 and sIL-2R levels were measured in triplicate by an ELISA assay (R&D System). Statistical analysis of the results was carried out using Student's *t* test. Correlation analysis was done by linear regression analysis and by Pearson's correlation coefficient.

RESULTS

Incorporation of 3H-thymidine

In this study, the mitogen PHA for T-cells stimulation was used. The immunosuppressive drugs used were PRED, CsA and the combination of these two drugs at different concentrations. The

inhibition of the proliferation of the activated T cells by CsA, PRED and by their combination was concentration dependent (Figure 1a). The greatest inhibition of proliferation was induced by PRED at the concentration of (25 µg/ml/10⁶ cells (p<0.001), (Figure 1a).

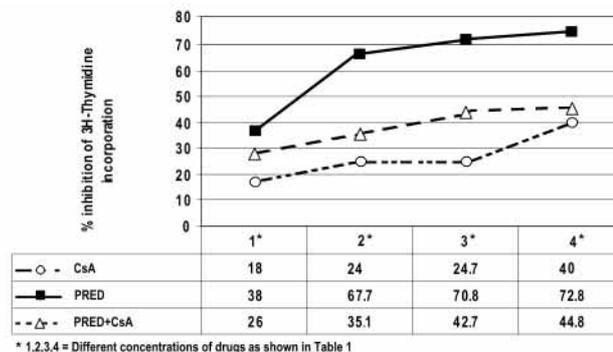


Figure 1a. The inhibition of the proliferation of activated T cells by CsA, PRED and CsA+PRED

We examined whether the suppressive activity of ara-C and L-asparaginase was concentration and time related on T cell activity. Both chemotherapeutic drugs inhibited PHA-stimulated T cells proliferation in concentration and time dependent manner (Figure 1b). For ara-C the greatest inhibition was induced after the exposure time of 15 minutes at the concentration of (8 µg/ml/10⁶cells (p<0.001) (Figure 1b) and for L-asparaginase after 18 hours at the concentration of 10 U/ml/10⁶cells (p<0.001) (Figure 1b).

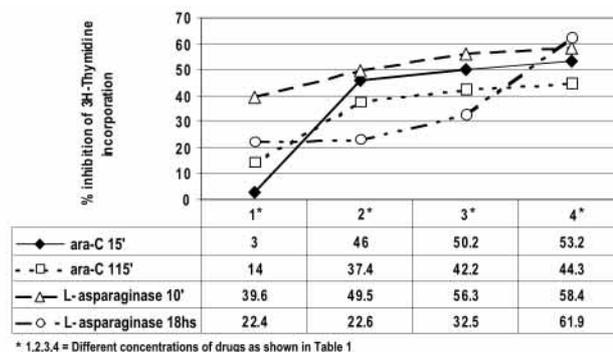


Figure 1b. The inhibition of the proliferation by the activated T cells by ara-C and L-asparaginase

Production of IL-2 by T cell

Different concentrations of the PRED, CsA, PRED + CsA, ara-C, L-asparaginase were added to the culture of PHA stimulated T cells, and the supernatants were tested for IL-2 activity 48 hours thereafter. As shown in Figures 2a, 2b and 2c all drugs strongly inhibited secretion of IL-2 after T cells stimulation in time and concentration-dependent manner. The greatest inhibition was caused by CsA ara-C at the concentration of 400 ng/ml/10⁶ cells (p<0.001), (Figure 2a). For ara-C, the corresponding greatest inhibition was obtained for exposure time of 115 minutes and concentration of 10 µg/ml/10⁶cells (p<0.001), (Figure 2b), while for L-asparagi-

nase for 10 minutes exposure and concentration of (6U/ml/10⁶cells (p<0.001), (Figure 2c).

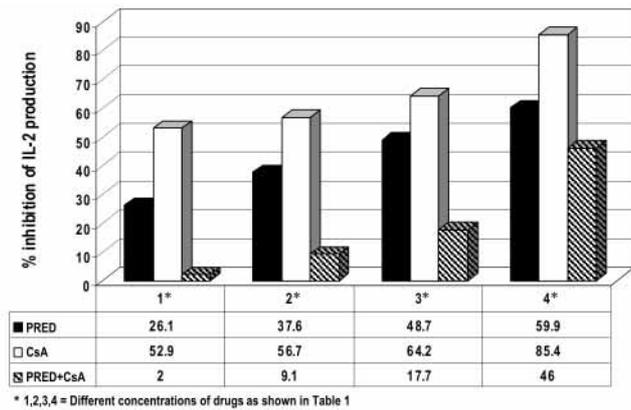


Figure 2a. The inhibition of IL-2 production from the activated T cells by CsA, PRED and CsA+PRED

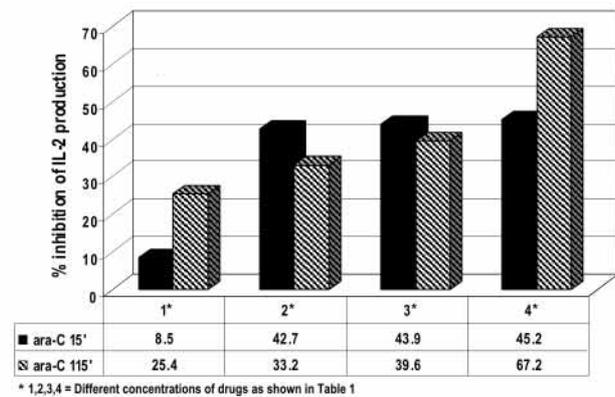


Figure 2b. The inhibition of the IL-2 production from the activated T cells by ara-C

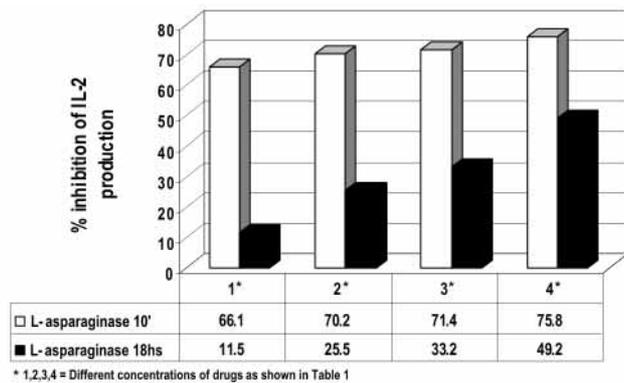


Figure 2c. The inhibition of the IL-2 production from the activated T cells by L-asparaginase

Production of sIL-2R

The supernatants from T cells cultures were also tested for sIL-2R activity. Different concentrations of the PRED, CsA, PRED+CsA, ara-C, L-asparaginase, were added to the culture of PHA stimulated T cells, and the supernatants were tested for sIL-2R activity 2 days later. As shown in Figures 3a, 3b and 3c all drugs strongly inhibited the secretion of sIL-2R after T cell stim-

ulation, in time and concentration-dependent manner. The greatest inhibition of the sIL-2R production from the activated T-cells was caused by CsA at the concentration of 400 ng/ml/10⁶cells (p<0.001), (Figure 3a). For ara-C, the corresponding greatest inhibition was for the exposure time of 15 minutes, at the concentration of ≥8 μg/ml/10⁶cells (p<0.001), (Figure 3b). For L-asparaginase, the greatest inhibition was obtained with 10 U/ml/10⁶cells (p<0.01), (Figure 3c) while there was no statisti-

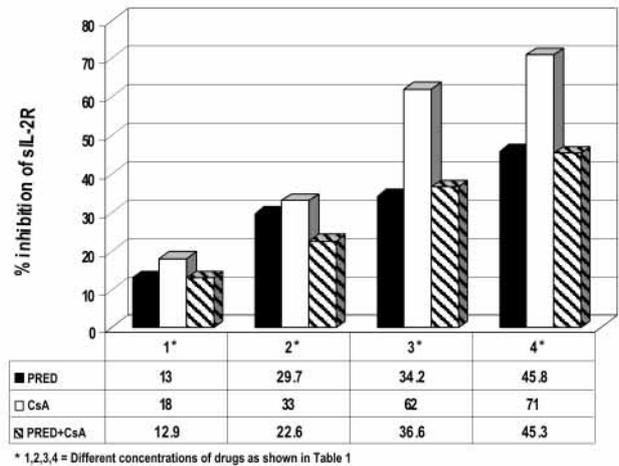


Figure 3a. The inhibition of the sIL-2R production from the activated T cells by CsA, PRED and CsA+PRED

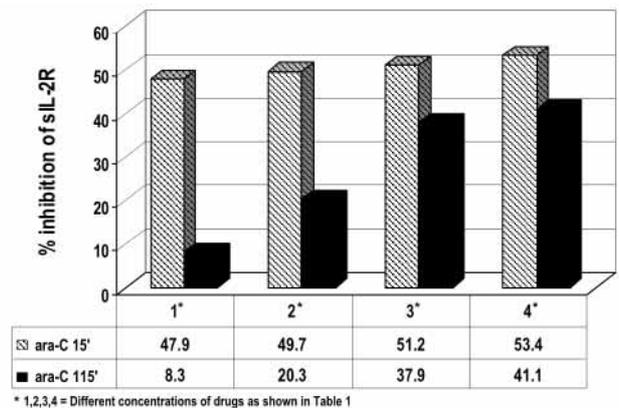


Figure 3b. The inhibition of the sIL-2R production from the activated T cells by ara-C

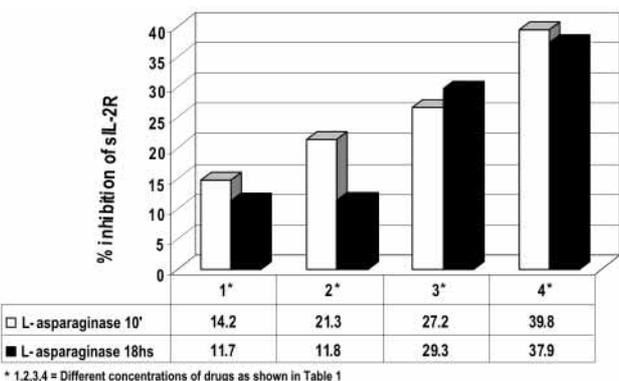


Figure 3c. The inhibition of the sIL production from the activated T cells by L-asparaginase

cal difference between the two exposure times of 10 minutes and 18 hours, respectively.

Effects of immunosuppressive and chemotherapeutic drugs on the expression of activation antigens

As shown in Table 2, PHA-stimulated T cells express proliferation antigens CD3, CD4 and CD8 as well as activation antigen CD25 (IL-2 receptor) on their membranes. All the immunoregulatory drugs inhibited the lymphocyte proliferation and expression of activation antigens.

Table 2. The mean values* of T cell receptors expression after treatment with immunoregulatory drugs

Receptor expression	A** Untreated	A**+PRED	A**+CsA	A**+(PRED+CsA)	A**+Ara-C 15'	A**+Ara-C 115'	A**+L-Asparaginase 10'	A**+L-Asparaginase 18h
CD2	90	82	82	79	79	79	80	79
CD3	75	60	66	61	50	48	52	50
CD4	51	40	32	31	25	24	25	24
CD8	31	37	33	30	23	22	23	21
CD25	10	7	2	4	2	2	3	2

* = Values are given as percent (%)
 A** = T cells activated with PHA

DISCUSSION

Triggering of the T cell receptor (TCR/CD3) complex stimulates a cascade of biochemical events that transduce the signal across the cell membrane and initiate intracellular processes leading to activation and proliferation (7).

PRED is one of the most potent and widely used immunosuppressive drugs. The mechanisms by which it suppresses the immune responses have been widely investigated (8-13). It affects the distribution, growth, differentiation and function of neutrophils, lymphocytes and monocytes (8,11). Corticosteroids can block the progression of PHA-stimulated lymphocytes through the mitotic cycle. They inhibit the entry of cells into the G1 phase and arrest the progression of activated lymphocytes from the G1 to the S phase (12).

In this study we demonstrated that PRED inhibits preactivated T cells by down-regulation of signal transduction through IL-2R. The inhibitory effect of PRED is due to postreceptor effects, as suggested by immunofluorescence analysis for cell surface IL-2R expression. These data expand previous observations that PRED inhibits the TCR-mediated induction of IL-2 gene transcription and suggest a "dual hit" mechanism for the PRED-mediated inhibition of T cells, whereby PRED inhibits both IL-2 production and cell activation (10,12). These observations provide a basis for the profound inhibitory effect of PRED on cell-mediated immunity.

CsA is a fungal metabolite that acts primarily on T lymphocytes. The effectiveness of CsA is based on selective inhibition of T helper cell activation and slight enhancement of T suppressor cell activity (14). The greatest effect on T helper cells is by the inhibition of interleukin-2 production. The decreased production of IL-2 in turn leads to decreased numbers of IL-2R (15,16). Because there is a positive feedback through IL-2 production and IL-2R, the decreased IL-2 production also leads to decreased IL-2R on T helper cells (17-19). The combination of CsA and PRED mitigates two adverse effects on T cells. It affects both lymphocyte proliferation and their activation (20). Cytosine arabinose (ara-C) is an antineoplastic drug (21) and it inhibits pyrimidine synthesis, through a complex pathway (22-25). The drug must undergo enzymatic conversion to the active form of cytosine triphosphate derivative (ara-CTP), and then binds competitively to inhibit DNA polymerase (26). In this study the toxicity of ara-C was studied on normal human peripheral blood PHA-stimulated lymphocytes cells *in vitro*. Clinically relevant ara-C concentrations were toxic against mitogen-stimulated blood lymphocytes. Concentration dependent effects included decreased DNA synthesis assessed by 3H-thymidine incorporation, inhibition of the production of new cells and antiproliferative effect. In fact, we demonstrated that ara-C significantly decreased its own uptake and DNA incorporation (24,26).

L-asparaginase hydrolyzes asparagine that is required for cell growth. Tumor cells have higher amounts of L-asparaginase in comparison to normal cells (21,27). The immunosuppressive property of the enzyme L-asparaginase is to inhibit lymphocyte blastogenesis. The immunosuppressive effects of L-asparaginase may be due to the capability of the enzyme to degrade L-glutamine (28). There is great potential of this enzyme to be used as immunosuppressive drug in the treatment of leukemia (28).

Our results demonstrate that inhibition of cellular DNA synthesis by cytosine arabinose was significantly related to the drug concentrations and exposure time (29- 31).

CONCLUSION

This study presented a series of experiments demonstrating that PRED, CsA, the combination of these two drugs, ara-C and L-asparaginase, inhibit the incorporation of 3H-thymidine, the induction of IL-2 synthesis as well as the secretion of sIL-2R from preactivated T lymphocytes. The study of activation marker expression by the fluorescence microscopy is a useful complement to traditional measurements of cell proliferation and activation as they yield subset-specific information about cellular processes, which precede lymphocyte proliferation and activation.

The immunoregulatory drugs we used inhibit lymphocyte prolifer-

ation and activation but in a different way: PRED affects mainly the lymphocyte proliferation, CsA affects activation and ara-C and L-asparaginase influence both proliferation and activation at approximately the same degree.

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