ANTIOXIDANTS & REDOX SIGNALING
Volume 4, Number 3, 2002

Forum Original Research Communication

L-Carnitine Reduces Lymphocyte Apoptosis and Oxidant Stress in HIV-1-Infected Subjects Treated with Zidovudine and Didanosine

SONIA MORETTI,[1] GIUSEPPE FAMULARO,[2] SONIA MARCELLINI,[1] ANTONIO BOSCHINI,[3] GINO SANTINI,[1] VITO TRINCHIERI,[1] LUCIANO LUCCI,[1] EDOARDO ALESSE,[1] and CLAUDIO DE SIMONE[1]

ABSTRACT

Apoptosis is critical to the progression of human immunodeficiency virus-1 (HIV-1) infection. It appears reasonable that antiretroviral therapies may not achieve a full control of the infection in the absence of an impact on apoptosis. We assigned 20 asymptomatic HIV-infected subjects with advanced immunodeficiency to receive either zidovudine (AZT), and didanosine (DDI) or the same regimen plus L-carnitine, a known antiapoptotic drug, for 7 months. Immunologic and virologic parameters were measured at baseline and after 15, 60, 120, and 210 days of treatment. We assessed on each time point the following: (a) the frequency of peripheral blood apoptotic CD4 and CD8 lymphocytes, CD4 and CD8 cells with disrupted mitochondrial membrane potential, and CD4 and CD8 cells undergoing oxidant stress; (b) the expression of the molecular markers of apoptosis Fas and caspase-1; and (c) the expression of p35/cdk-5 regulatory subunit that is involved in regulating cell survival and apoptosis. Absolute CD4 and CD8 counts and plasma viremia were also measured. Apoptotic CD4 and CD8 cells, lymphocytes with disrupted mitochondrial membrane potential, and lymphocytes undergoing oxidant stress were greatly reduced in subjects treated with AZT and DDI plus L-carnitine compared with those who did not receive L-carnitine. Fas and caspase-1 were down-expressed and p35 overexpressed in lymphocytes from patients of the L-carnitine group. No difference was found in CD4 and CD8 counts and viremia between the groups. No toxicity of L-carnitine was recognized. The addition of L-carnitine is safe and allows apoptosis and oxidant stress to be greatly reduced in lymphocytes from subjects treated with AZT and DDI. *Antioxid. Redox Signal.* 4, 391–403.

INTRODUCTION

THE PROGRESSION OF INFECTION with the human immunodeficiency virus (HIV) is associated with an inappropriate apoptosis of lymphocytes that is thought to contribute substantially to the immune impairment and decline in CD4 counts (1–6). A correlation between the degree of apoptosis and disease evolution has been indeed observed (3, 4, 25, 26, 30, 45), and long-term nonprogressors, who remain healthy and immunocompetent over an extended time despite active HIV infection, have a low degree of apoptosis as compared with subjects progressing to the acquired immunodeficiency syndrome (AIDS) (3, 35).

Based on these findings, it is conceivable that antiviral therapy, even with highly active drugs, may not achieve a full control of the infection in the absence of a relevant impact on the process of lymphocyte apoptosis, irrespectively of whether plasma viremia is lowered to undetectable levels. We have recently found that the initiation of combined antiviral therapy had no impact on the process of lymphocyte apopto-

[1]Department of Experimental Medicine, University of L'Aquila; [2]Department of Medical Sciences, San Camillo Hospital, Rome; and [3]Community of San Patrignano, Rimini, Italy.

sis in a cohort of drug-naive patients, while promptly reducing the viral load and improving CD4 counts (38). It is also of interest that antiretroviral drugs, such as zidovudine (AZT), may trigger both the morphological and biochemical changes typical of apoptosis in cells not infected with HIV (47). Furthermore, no differences in the frequency of apoptotic lymphocytes have been found in relation to AZT therapy (38).

In our opinion, this background raises the hypothesis that any regimen of antiviral therapy can no longer maintain the homeostasis between HIV replication and CD4 cell production if the unregulated apoptosis of lymphocytes persists. In turn, the combination of active antiretroviral therapy with effective antiapoptotic strategies could offer a crucial tool in the attempt to eradicate the infection completely and restore immune competence.

The clinical potential of carnitines as an antiapoptotic strategy in the treatment of HIV-1-positive patients has been recently highlighted. Carnitines have appreciable antiapoptotic properties, when tested *in vitro* (16, 21), and are frequently deficient in untreated HIV-positive patients (11, 12) or in AIDS patients with clinically manifest neuropathy (23). Pilot clinical trials have recently demonstrated that the carnitine treatment significantly reduces *in vivo* the rate of lymphocyte apoptosis in subjects infected with HIV (8, 37). This has been attributed to the down-modulation of transduction signals involved in the apoptotic response (*i.e.,* ceramide generation) (8, 16, 37) or organelle function (i.e. mitochondria pore transition state) (9) and also to the activation of the growth hormone (GH)–insulin-like growth factor (IGF)-1 axis (17).

In this present study, we provide evidence that the addition of L-carnitine to a double-drug antiretroviral regimen [AZT plus didanosine (DDI)] results in the following: (a) a reduction in the frequency of apoptotic CD4 and CD8 lymphocytes, (b) a decline in the frequency of CD4 and CD8 cells with disrupted mitochondrial membrane potential, a marker of the irreversible commitment to undergo apoptosis. The frequency of cells with mitochondrial alterations on flow cytometry indicating oxidant stress was also reduced, and (c) a decline in the frequency of CD4 and CD8 lymphocytes expressing molecular markers of apoptosis, such as Fas and caspase-1. By contrast, subjects receiving only treatment with AZT and DDI, without the addition of L-carnitine, had no improvement in those markers of apoptosis.

MATERIALS AND METHODS

Patients and treatment

Twenty male subjects, living in the Community of San Patrignano (Rimini, Italy), with symptomless HIV infection, advanced immunodeficiency, and without any previous antiretroviral treatment (mean age, 34.8 ± 2.2 years; mean CD4 cell counts, 295 ± 147 cells/μl, range 50–595) were enrolled in the study after they gave written informed consent. The study design was approved by the internal ethical committee of S. Patrignano. Only men were enrolled because carnitine levels in the blood vary according to sex (15). The patients had stable CD4 counts until the last 12 months of follow-up, when progressively declining counts were revealed. Nutritional status was defined as medium-good under clinical conditions, and all patients either had stable body weight or had maintained their weight within a 5% variation range during the previous 4 months, and had albumin levels above 4 g/dl and total carnitine levels comparable to those in healthy individuals. Persistent diarrhea was not reported in any patient in the previous 6 months, and nutritional support was not requested. None had clinical or laboratory evidence of kidney dysfunction, and the Karnofsky score was >90 in all of them. None of them had any AIDS-defining condition over the entire study period.

Other main eligibility criteria were a hemoglobin concentration of >90 g/L, a total neutrophil count of $>10^9$/L, a platelet count of $>25 \times 10^9$/L, serum transaminase levels no higher than six times the upper limit of normal, serum amylase values within 1.25 times the upper limit of normal, age >18 years, and a Karnofsky index value of >80.

The main exclusion criteria were previous treatment with other antiretroviral drugs, severe diarrhea unresponsive to treatment, a history of or current pancreatitis, bilateral moderate peripheral neuropathy, the need for neurotoxic or cytotoxic therapies, and transfusion in the 30 days preceding the investigation.

The patients were treated with combined AZT and DDI for 7 months. The assigned dosage of AZT (Wellcome, Beckenham, U.K.) was 12 mg/kg/day (range 500–1,000), and DDI (Bristol–Myers, Squibb, Wallingford, CT, U.S.A.) was assigned on a weight-adjusted basis: a 375- and 250-mg sachet was given twice daily to patients weighing more than and less than 60 kg, respectively. Moreover, 10 patients were randomly assigned through computer-generated numbers to receive also highly purified L-carnitine (Carnitene, Sigma Tau, Pomezia, Italy), *per os*, 6 g/day for the study period.

To evaluate several immunologic and virologic parameters, samples of peripheral blood were taken at baseline (T0), on day 15 (T1), on day 60 (T2), on day 120 (T3), and on day 210 (T4). Blood was collected in EDTA tubes and kept on ice until cell preparation and analysis.

Peripheral blood mononuclear cell (PBMC) isolation

PBMCs were separated by Lymphoprep gradient centrifugation (Nycomed, Oslo, Norway), washed twice with phosphate-buffered saline (PBS), and maintained in complete culture medium at 4°C until labeling. In addition, aliquots of cells were incubated for 18 h in RPMI 1640 (Life Technologics, Inc., Paisley, U.K.) supplemented with 5% heat-inactivated fetal calf serum (Life Technologics), 1 m*M* L-glutamine (Life Technologics), 10 m*M* HEPES (Sigma Chemical Company, St. Louis, MO, U.S.A.), and 10 IU/ml penicillin/streptomycin (Life Technologics) at 37°C in a 5% CO_2-humidified atmosphere, and then used for flow cytometry analysis of apoptosis.

Expression of surface and intracellular antigens

The absolute counts of cells bearing the CD4 and CD8 surface phenotype were determined by standard methods. In brief, PBMCs were stained with an anti-CD4 and an anti-

CD8 monoclonal antibody (mAb) labeled with fluorescein isothiocyanate (FITC), according to the manufacturer's instructions (Becton–Dickinson Immunocytometry Systems, San Jose, CA, U.S.A.).

To evaluate the intracellular expression of caspase-1 and p35, freshly isolated PBMCs, adjusted at a concentration of 1×10^6 cells/ml, were added with 1 ml of 0.25% paraformaldehyde, while vortexing at 300 g for 5 min, and then incubated in the dark for at least 15 min at room temperature. After washing with PBS, the samples were added with 1 ml of cold (4°C) 70% methanol and incubated in the dark for 60 min at 4°C in order to make the cell and nuclear membrane permeable. After washing with PBS, the samples were incubated with anti-caspase-1 mAb (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) or anti-p35 mAb (Santa Cruz Biotechnology) and anti-rabbit IgG FITC conjugate (Sigma Chemical Company) in the dark for 30 min at 4°C. Then, after two washings with PBS containing 2% fetal calf serum, the samples were analyzed by flow cytometry.

Assessment of cells undergoing apoptosis

Phenotypic analysis of apoptotic T cells. Quantification and phenotypic analysis of apoptotic cells from the short-term cultured lymphocytes was performed by staining apoptotic cells with 7-aminoactinomycin D (7-AAD; Sigma) as reported by Schmid *et al.* (48). This method was shown to discriminate between early and late apoptotic cells due to their increased membrane permeability. Cultured lymphocytes were first incubated with FITC-conjugated mAbs to surface antigens as described above, and washed cells were then incubated with 20 μg/ml 7-AAD for 20 min at 4°C protected from light. Stained cells were further fixed with 1% paraformaldehyde in PBS in the presence of 20 μg/ml of non-fluorescent actinomycin D (Sigma) to block 7-AAD staining within apoptotic cells and avoid nonspecific labeling of living cells. Finally, the double-stained cells were incubated overnight at 4°C in the dark and were then analyzed in their staining solution by a FACScan flow cytometer (Becton–Dickinson). The spectral properties of 7-AAD allow the staining of apoptotic cells by fluorescence emission in the red channel FL-3 (650 nm < wavelength < 850 nm), and the easily and simultaneous labeling of cell-surface antigens (FL1 and FL2). Scattergrams were generated by combining foward scatter (FSC) with 7-AAD fluorescence, and regions were drawn around clear-cut populations having negative (live cells), dim (early apoptotic cells), and bright fluorescence (late apoptotic cells). A minimum of 10,000 events was collected on each sample.

Staining of apoptotic nuclei with propidium iodide (PI). Lymphocyte apoptosis was quantified as the percentage of cells with hypodiploid DNA using the technique of Nicoletti *et al.* (40). In brief, following a short-term culture, cell suspensions were centrifuged at 200 *g* for 10 min. For staining of surface antigens, aliquots of 1×10^6 cells were incubated with appropriately titered phenotype FITC-conjugated mAbs as previously described and, after washing, the pellet was gently resuspended in 1 ml of hypotonic fluorochrome solution (50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100, 0.05 mg/ml RNase A; Sigma). Cells were kept overnight at 4°C, then analyzed on the FACScan flow cytometer in their staining solution. Apoptotic nuclei appeared as a broad hypodiploid DNA peak that was easily discriminable from the narrow peak of nuclei with normal (diploid) DNA content in the red fluorescence channel. Orange PI fluorescence was collected after a 585/42 nm bandpass filter and was displayed on a four-decade log scale. Acquisition on the flow cytometer was done in the low sample flow rate setting (12 μl/min) to improve the coefficient of variation on the DNA histograms. Lymphocytes were gated on the basis of their physical parameters FSC and side scatter (SSC), for exclusion of debris and clumps, and a minimum of 10,000 events was collected on each sample.

Analysis of mitochondrial functions. To evaluate the mitochondrial transmembrane potential ($\Delta\Psi_m$), cells (5×10^5/ml) were incubated for 15 min at 37°C with 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$; 40 n*M* in PBS; Molecular Probes, Eugene, OR, U.S.A.] (43). Control experiments were performed in the presence of 5 μ*M* carbamoyl cyanide *m*-chlorophenylhydrazone (15 min, at 37°C), an uncoupling agent that abolishes the $\Delta\Psi_m$.

Intracellular hydrogen peroxide (H_2O_2) generation was measured by 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes Inc.; 5 μ*M* in PBS; 1 h, 37°C) (24). For DCFH-DA, a positive control (cells kept 2 min in 15 m*M* H_2O_2 and washed three times) was inserted.

Analysis were performed on a FACScan cytofluorometer (Becton–Dickinson). FSC and SSC were gated on the major population of normal-sized lymphoid cells.

Plasma polymerase chain reaction (PCR) for HIV RNA determination

Peripheral blood was collected from each subject in tubes containing acid-citrate dextrose and processed within 6 h. Plasma was separated from whole blood by centrifugation and stored in liquid nitrogen. We used 200 μl of plasma to estimate the HIV RNA copies per milliliter. The application of quantitative PCR was performed using the Amplicor detection system (Roche, Branchburg, NJ, U.S.A.) and the gene AMP 9600 (Perkin–Elmer, Norwalk, CT, U.S.A.) thermal cycler. In brief, the HIV RNA was extracted from each sample using guanidium, reverse-transcribed, amplified by rTh DNA polymerase, and detected using a method based on changes in optical density produced by reactions mediated by horseradish peroxidase. A noncompetitive, internal control was introduced to monitor sample and reaction interference.

Safety of treatment

During the trial, a complete physical examination was performed and blood counts and a biochemical profile obtained at baseline and then, regularly, each week throughout the entire study period. Patients were ranked on the Karnofsky performance score, by the same investigator, on each visit over the course of the trial.

Statistical analysis

The Kolmogorov–Smirnov two-sample test was used to prove the homogeneity of the two groups of patients. Student's *t* test was used for the statistical analysis of results

TABLE 1. CD4 AND CD8 COUNTS, HIV VIREMIA, AND EXPRESSION OF CASPASE-1 (ICEP20), P35, AND FAS IN HIV-INFECTED PATIENTS TREATED WITH ANTIRETROVIRALS OR ANTIRETROVIRALS PLUS L-CARNITINE

	CD4		*CD8*		*CD4/CD8*	*ICEp20*	
	%	*n*	*%*	*n*	*ratio*	*%*	*n*
Antiretrovial therapy (n = 10)							
T0	13.61 ± 5.1	242 ± 122	65.07 ± 10.3	1,259 ± 888	0.22 ± 0.11	25.33 ± 13.1	423 ± 242
T1	14.54 ± 6	294 ± 147	65.28 ± 11	1,411 ± 990	0.24 ± 0.13	25.56 ± 13.6	503 ± 321
T2	15.31 ± 6.7	298 ± 132	62.59 ± 10.4	1,236 ± 518	0.26 ± 0.15	28.08 ± 12.8	529 ± 283
T3	17.43 ± 6.8	330 ± 151	60.9 ± 9.2	1,157 ± 528	0.31 ± 0.16	33.33 ± 15.8	632 ± 360
T4	17.65 ± 7.6	329 ± 173	63.21 ± 11.2	1,160 ± 490	0.31 ± 0.2	34.95 ± 13.9	629 ± 319
Antiretroviral therapy + L-carnitine (n = 10)							
T0	15.96 ± 7.7	348 ± 152	61.39 ± 9.8	1,485 ± 620	0.28 ± 0.16	26.23 ± 6.3	609 ± 211
T1	17.02 ± 7.1	367 ± 206	62.72 ± 8.4	1,470 ± 783	0.29 ± 0.14	20.31 ± 6.7	464 ± 249
T2	18.21 ± 8	413 ± 226	59.53 ± 8.6	1,413 ± 662	0.32 ± 0.16	20.08 ± 4.7	482 ± 233
T3	20.29 ± 8.6	452 ± 231	57.91 ± 8.9	1,353 ± 637	0.38 ± 0.19	19.44 ± 5.5	455 ± 215
T4	19.85 ± 7.5	443 ± 220	59.47 ± 7.1	1,404 ± 616	0.35 ± 0.15	19.28 ± 4.8	442 ± 182

See Materials and Methods for details.

from each later time point and T0 between the two groups. The statistically significant differences ($p < 0.05$) were confirmed by the nonparametric test of Mann–Whitney. The one-way analysis of variance was used to compute each of the several parameters of interest within both groups of study, and the statistically significant differences, calculated from each later time point and T0 by a multiple range test, were performed with a paired Student's t test.

RESULTS

Lymphocyte apoptosis

Lymphocyte apoptosis was investigated using 7-AAD, a fluorescent DNA-intercalating agent that penetrates only the apoptotic cells because of their altered membrane integrity. Using this method, we found that the treatment with AZT and DDI had no impact on the frequency of apoptotic CD4 and CD8 lymphocytes (Tables 1 and 2). There was no significant difference between each time point compared with baseline.

Subjects treated with combined antiretroviral therapy plus L-carnitine had a significant reduction in the frequency of apoptotic CD4 lymphocytes on T4 compared with the group receiving only AZT and DDI ($p < 0.031$) (Tables 1 and 2 and Fig. 1). This effect occurred gradually between each time point and baseline. A similar trend toward a decline in the frequency of apoptotic cells was observed in this group also among CD8 lymphocytes, although the difference did not reach statistical significance.

Representative flow cytometry profiles are shown in Fig. 1. There was a strong reduction in the frequency of CD4 and CD8 cells stained by 7-AAD (*i.e.,* apoptotic lymphocytes) in the group of patients who received AZT and DDI plus

TABLE 2. FREQUENCY OF APOPTOTIC CD4 AND CD8 LYMPHOCYTES AND CD4 AND CD8 LYMPHOCYTES WITH IMPAIRED MITOCHONDRIAL METABOLISM AND INCREASED SUPEROXIDE GENERATION

	7-AAD				*PI*			
	CD4%	*CD4n*	*CD8%*	*CD8n*	*CD4%*	*CD4n*	*CD8%*	*CD8n*
Antiretroviral therapy (n = 10)								
T0	29.26 ± 10.3	71 ± 42	17.28 ± 7.7	193 ± 117	29.2 ± 9.8	553 ± 387	17.18 ± 7.5	294 ± 164
T1	28.51 ± 11.4	80 ± 43	19.79 ± 9.5	241 ± 127	28.27 ± 11.2	581 ± 372	19.69 ± 8.9	375 ± 176
T2	25.31 ± 11.7	79 ± 51	16.46 ± 10.2	197 ± 140	25.45 ± 11.6	511 ± 329	16.34 ± 9.2	311 ± 204
T3	26.59 ± 14.8	99 ± 78	19.12 ± 9.5	200 ± 106	26.84 ± 14.4	535 ± 385	19.05 ± 8.7	331 ± 167
T4	28.21 ± 11.7	99 ± 62	17.07 ± 6	192 ± 98	28.17 ± 11.4	542 ± 333	17.07 ± 5.8	313 ± 145
Antiretroviral therapy + L-carnitine (n = 10)								
T0	34.46 ± 16	104 ± 47	20.99 ± 9	295 ± 144	34.3 ± 15.4	769 ± 338	21.06 ± 9.4	478 ± 220
T1	32.45 ± 17.1	101 ± 49	20.88 ± 12.3	297 ± 214	32.43 ± 16.2	732 ± 419	20.81 ± 11.5	461 ± 288
T2	25.29 ± 17.2	88 ± 72	15.76 ± 10.3	213 ± 177	25.24 ± 16.4	541 ± 349	15.69 ± 9.5	345 ± 233
T3	18.68 ± 14	66 ± 45	13.52 ± 9.7	174 ± 146	18.78 ± 13.1	409 ± 298	13.57 ± 8.7	291 ± 200
T4	17.11 ± 8.1	65 ± 32	14.27 ± 8.1	181 ± 103	17.14 ± 7.5	373 ± 168	14.26 ± 7.2	303 ± 143

See Materials and Methods for details.

TABLE 1. (CONTINUED)

p35		*Fas*		*HIV*
%	*n*	*%*	*n*	*n*
37.47 ± 14.6	725 ± 641	37.55 ± 14.1	692 ± 381	59,558 ± 34,627
37.85 ± 11.7	823 ± 598	35.91 ± 12	731 ± 361	4,549 ± 4,001
39.52 ± 6	775 ± 300	38.29 ± 11.6	771 ± 378	2,943 ± 1,563
39.63 ± 6.7	755 ± 344	34.94 ± 8.2	645 ± 247	2,757 ± 2,314
38.73 ± 5.9	714 ± 312	32.17 ± 5.6	588 ± 229	4,272 ± 3,022
33.4 ± 14.6	857 ± 679	42.83 ± 11	985 ± 331	162,606 ± 252,991
38.56 ± 15.4	921 ± 749	35.12 ± 13.5	812 ± 550	3,262 ± 1,451
41.38 ± 9.6	1,017 ± 715	34.83 ± 8.2	813 ± 388	2,427 ± 2,391
44.21 ± 6	1,045 ± 604	34.53 ± 8.4	784 ± 341	4,557 ± 4,768
46.29 ± 6.8	1,087 ± 568	30.17 ± 8.2	672 ± 249	64,931 ± 70,321

L-carnitine, but this reduction was not observed in subjects treated with AZT and DDI only.

Results of the analysis of lymphocyte apoptosis by using the method with PI staining were comparable to those obtained using the 7-AAD method (Tables 1 and 2 and Fig. 2).

Identification of apoptotic lymphocytes was assessed also by considering the scatter characteristics of cells, based on the evidence that apoptotic cells can be easily distinguished from viable cells through the measurement of FSC and SSC light parameters, which are proportional to cell diameter and internal granularity, respectively (18). Indeed, whereas living cells display relatively high FSC/low SSC parameters, cells undergoing apoptosis shift to a lower FSC/high SSC compartment, consistent with the cellular changes occurring during the process of apoptosis (reduction of cell size and cytoplasmic volume and chromatin condensation) (18, 34). The results of this investigation were comparable to the results obtained by the 7-AAD and PI staining methods (data not shown).

Mitochondrial activity

The low incorporation of $DiOC_6(3)$, a cationic lipophilic fluorochrome that allows for the assessment of $\Delta\Psi_m$, is thought to reflect the disruption of the normal $\Delta\Psi_m$, an early and irreversible step in the effector phase of apoptosis (33). We found that peripheral blood T lymphocytes from HIV-positive donors incorporated at baseline low levels of $DiOC_6(3)$ compared with HIV-negative subjects. This indicates the irreversible commitment of those cells to undergo apoptosis.

The treatment with antiretrovirals had no impact on the frequency of $DiOC_6(3)^{low}$ cells over the study period (Tables 1 and 2). Even the frequency of lymphocytes labeled using DCFH-DA, a fluorochrome that detects H_2O_2 generation, was

TABLE 2. (CONTINUED)

$DiOC_6(3)$		*DCFH-DA*	
%	*n*	*%*	*n*
31.39 ± 13.2	524 ± 222	16.18 ± 7	260 ± 97
29.62 ± 13	574 ± 269	18.46 ± 7.3	339 ± 115
31.4 ± 18.3	551 ± 236	21.03 ± 9.2	379 ± 152
31.6 ± 18	530 ± 191	19.74 ± 6.8	358 ± 152
37.07 ± 11.8	620 ± 134	20.74 ± 5.6	360 ± 107
31.94 ± 14.9	755 ± 423	19.83 ± 6.6	457 ± 189
30.62 ± 12.1	678 ± 344	16.56 ± 7.2	347 ± 162
31.55 ± 15.3	733 ± 486	16.11 ± 9.8	349 ± 204
23.36 ± 12.7	517 ± 359	14.91 ± 8.4	339 ± 208
20.41 ± 5.3	476 ± 278	14.4 ± 6	317 ± 153

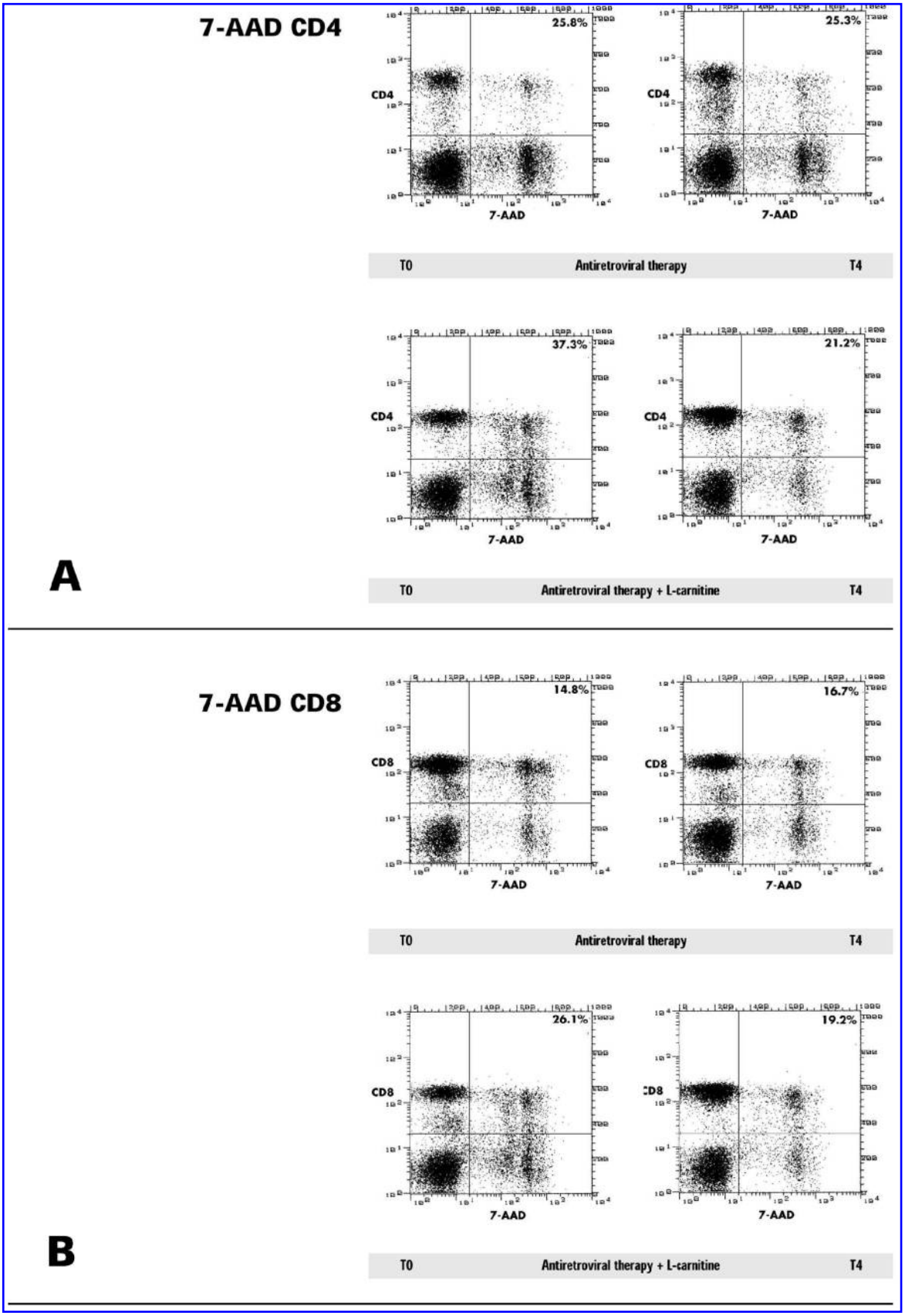

FIG. 1. Assessment of apoptotic peripheral blood CD4 and CD8 lymphocytes from subjects receiving AZT and DDI or AZT and DDI plus L-carnitine at baseline (T0) and after 7 months of therapy (T4). Two-color staining was used for the simultaneous determination of apoptosis by staining apoptotic cells with 7-AAD (**A** and **B**) and CD4 and CD8 surface phenotype, as described in Materials and Methods. Numbers refer to the percentage of apoptotic cells among the CD4 and CD8 lymphocytes, respectively. Results are representative of two independent experiments, each performed on two different subjects.

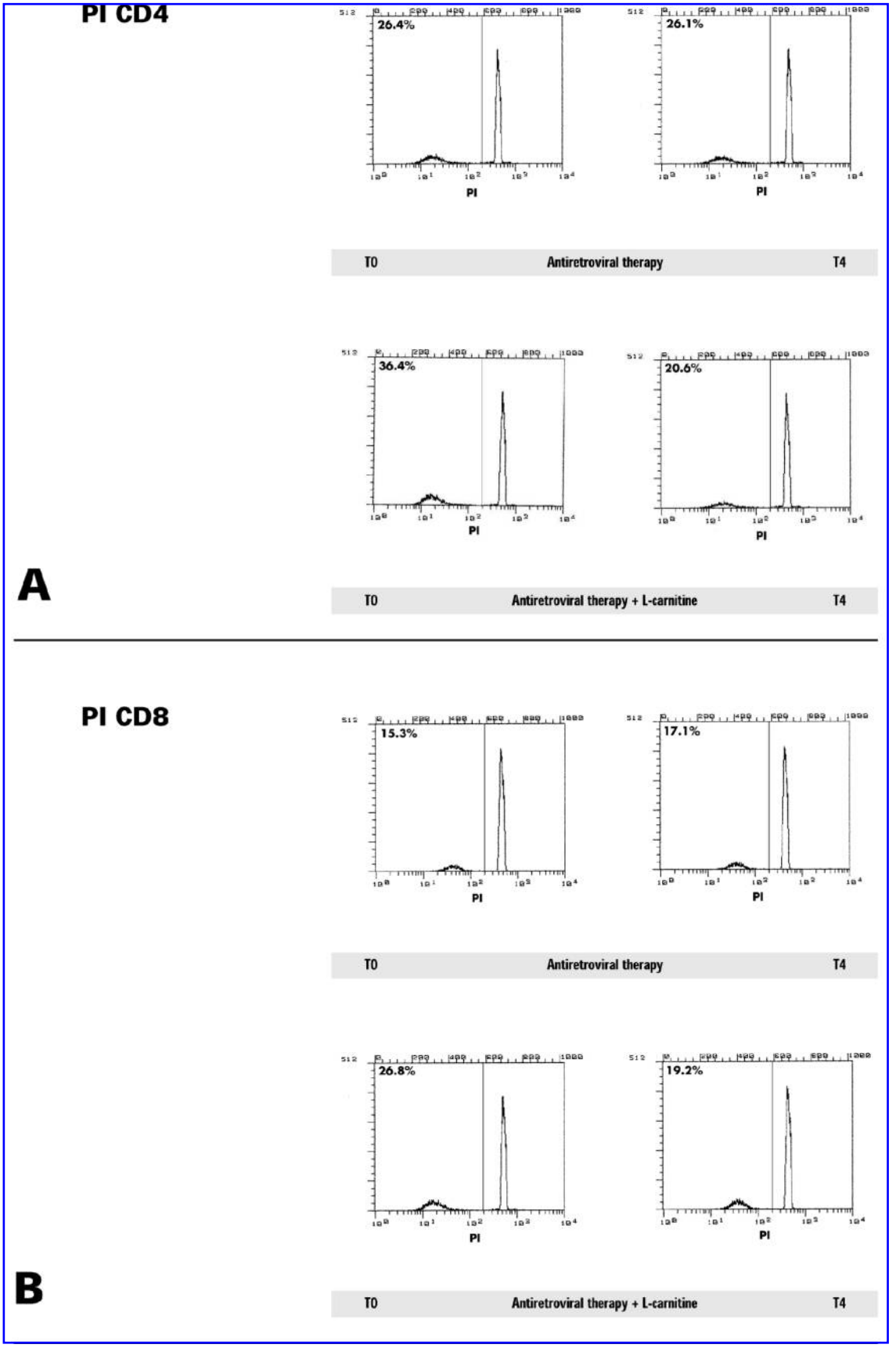

FIG. 2. Flow cytometry profiles of apoptotic CD4 (A) and CD8 (B) lymphocytes, detected using the PI staining method, at baseline (T0) and at the end of treatment period (T4) in subjects treated with AZT and DDI or AZT and DDI plus L-carnitine. Numbers refer to the percentage of apoptotic cells among the CD4 and CD8 lymphocytes, respectively. Results are representative of two independent experiments, each performed on two different subjects.

not significantly affected by AZT and DDI treatment in this group of patients, and rather a trend toward increasing frequency of $DiOC_6(3)^{low}$ and DCFH-DA^{high} lymphocytes was observed (Tables 1 and 2).

By contrast, patients receiving combined AZT and DDI plus L-carnitine showed a significant decline in the $DiOC_6(3)^{low}$ and DCFH-DA^{high} lymphocyte subsets at T4 compared with the other group ($p < 0.0011$ and $p < 0.031$, for the two parameters, respectively) (Tables 1 and 2).

Representative flow cytometry profiles are shown in Fig. 3.

Fas-positive and caspase-1-positive cells

The treatment with antiretrovirals had no impact on the counts of Fas-positive cells, and unchanged values were indeed measured at each time point compared with baseline (Tables 1 and 2). Even the counts of caspase-1-expressing cells were not significantly affected by the treatment in this group of patients and, rather, a trend indicating a progressive increase in the frequency of cells expressing caspase-1 was observed.

By contrast, subjects treated with combined AZT and DDI plus L-carnitine had a gradual decline in the frequency of Fas-positive and caspase-1-positive cells between each time point and baseline, and a significantly lower frequency of caspase-1-positive cells was detected at T4 compared with the group of patients receiving only antiretrovirals ($p < 0.005$) (Tables 1 and 2).

p35-positive lymphocytes

Given the enhanced susceptibility of CD4 and CD8 lymphocytes from HIV-infected subjects to undergo programmed cell death, we have analyzed the intracellular expression of p35 protein. No significant differences in p35 expression by lymphocytes was detected between the two groups of patients at baseline.

The combined treatment with antiretrovirals plus L-carnitine was paralleled by a significant increase in the frequency of p35-expressing cells at T4 with respect to the group of patients receiving AZT and DDI only ($p < 0.020$) (Tables 1 and 2). This increase in the frequency of p35-expressing cells was evident at each time point compared with the baseline.

CD4 and CD8 counts

A trend of increasing absolute CD4 counts was observed at each time point in either group of patients. At the end of the study period, one patient had an increase of >100%, one patient of 50%, and four patients of >30% over the baseline CD4 counts among subjects treated with AZT and DDI. The remaining four patients had a slight increase compared with the baseline (Tables 1 and 2). Likewise, among patients treated with combined AZT and DDI plus L-carnitine, four subjects had an increase of ~50%, one of >30%, and three had a slight increase compared with the baseline. In the remaining two patients, a marginal increase in CD4 counts was found at the end of the study period. However, in two of these subjects, slightly increased CD4 counts were observed on T1 and T3 and on T2 and T3, respectively (Tables 1 and 2).

Viral load

After initiation of antiviral therapy, there was a prompt reduction in plasma viral load, irrespective of whether patients received also L-carnitine or not. The large variability in baseline levels of HIV RNA reflects the differing viral burdens among patients (range, 20,174–135,900 and 4,381–877,000 copies/ml for the group treated with AZT and DDI only and the group treated with combined antiretrovirals plus L-carnitine, respectively). All subjects showed decreased levels compared with the baseline concentrations at each time point over the entire study period, although statistically significant differences were observed only in the group that did not receive L-carnitine (Tables 1 and 2) ($p < 0.02$ at each measurement).

Safety of treatment

No toxicity related to antiretrovirals or L-carnitine was observed, and none of the subjects investigated required reductions in the dose of drugs. In addition, even though we could not observe any significant improvements in the Karnofsky scores compared with baseline (all patients indeed had a Karnofsky score of >90 at enrollment), all the subjects treated with L-carnitine reported, with no exception, a sense of improved well-being by the second week of therapy.

DISCUSSION

Apoptosis is increased in lymph nodes and peripheral blood lymphocytes of HIV-infected individuals in both the CD4 and CD8 subsets of T cells (3, 4, 25, 26, 30, 45). In animal models of the disease, the numbers of apoptotic cells correlate with *in vivo* pathogenicity, and some studies, also in humans, have shown a correlation between the extent of apoptosis and disease progression (3, 4, 13, 25, 26, 30, 35, 45).

In this pilot study, we provide evidence that treatment with a two-drug antiretroviral regimen with AZT and DDI did not affect the process of lymphocyte apoptosis despite significantly improving the counts of CD4 cells and reducing HIV-1 viremia to almost undetectable levels. However, in subjects who received AZT and DDI combined with L-carnitine, we found a significant reduction in the frequency of apoptotic CD4 and, to a lesser degree, CD8 lymphocytes compared with the group treated only with AZT and DDI. At the end of the study period (T4), the difference in the frequency of apoptotic CD4 cells between the two groups of patients was strongly significant. Subjects who received antiretrovirals plus L-carnitine showed a progressive decline in the frequency of apoptotic cells that is evident when looking at the mean values at each time point. However, changes in actual values of apoptotic CD4 lymphocytes between each time point and baseline did not reach the statistical significance, probably because of the small size of the patient sample and the elevated standard deviation.

Both group of patients had a comparable increase in CD4 cell counts, although apoptosis of CD4 lymphocytes was significantly suppressed only in the L-carnitine group. This discrepancy needs to be fully investigated in additional studies. One possible explanation is that the suppression of HIV replication, rather than of apoptosis, is the major determinant required for observing an increase in absolute CD4 counts. Furthermore, the patients enrolled in this study were followed up only for 7 months, and we reasonably expect to find a difference with more extended follow-up. Preliminary data seem to be in agreement with this hypothesis.

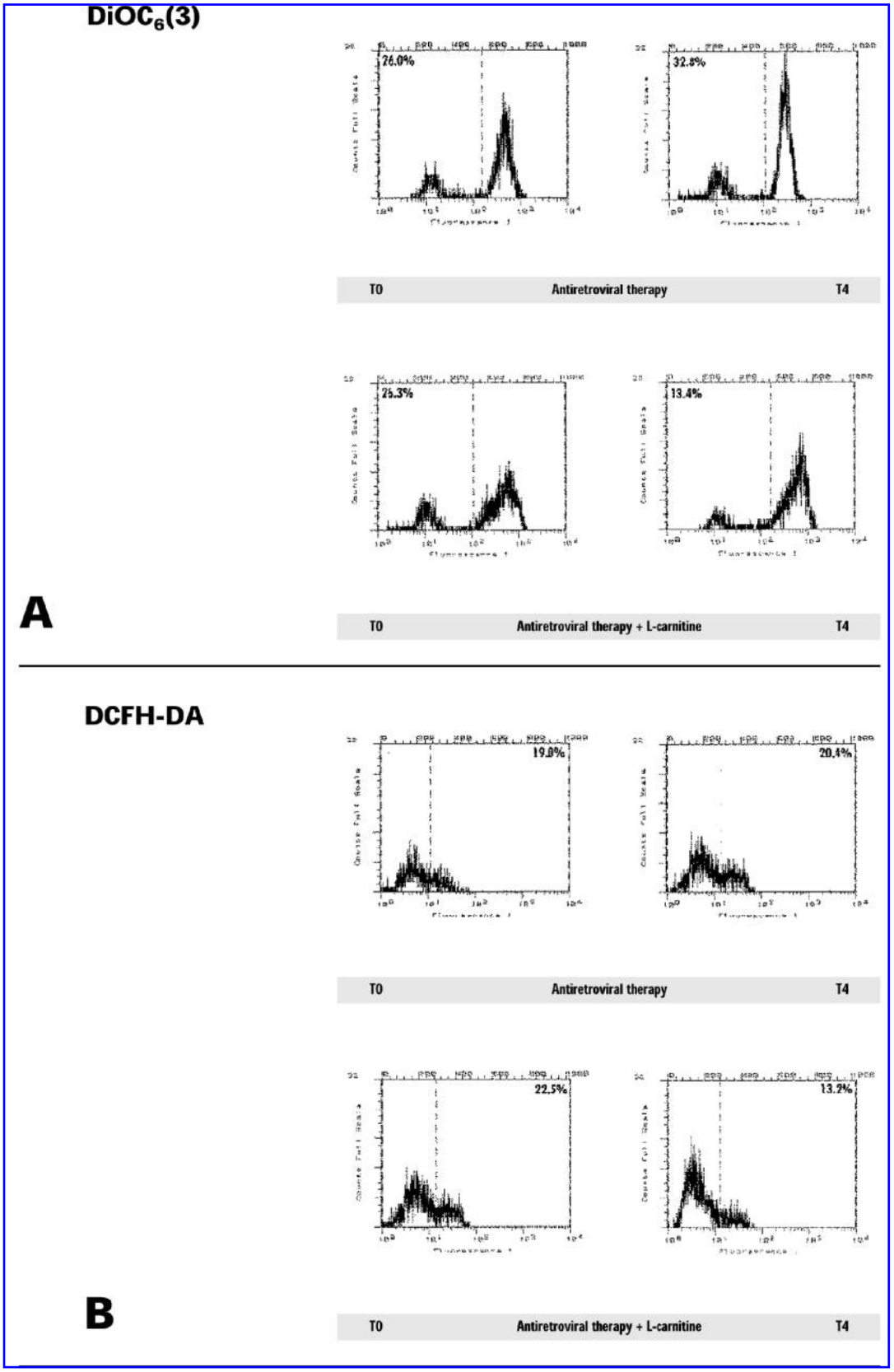

FIG. 3. Assessment of mitochondrial transmembrane potential ($\Delta\Psi_m$) and H_2O_2 generation in peripheral blood T lymphocytes from patients at baseline (T0) and after therapy (T4). (**A**) Determination of mitochondrial $\Delta\Psi_m$. Cells from two representative donors were labeled as described in Materials and Methods using a marker for transmembrane potential [$DiOC_6(3)$]. Numbers refer to the percentage of lymphocytes with disrupted $\Delta\Psi_m$. (**B**) Determination of H_2O_2 generation. Cells from two representative donors were labeled as described in Materials and Methods using a marker for H_2O_2 generation (DCFH-DA). Numbers refer to the percentage of DCFH-DA-positive lymphocytes.

Our assay of apoptotic lymphocytes was performed using flow cytometry to investigate cell staining with 7-AAD, a fluorescent DNA-intercalating agent that penetrates only the apoptotic cells because of their altered membrane permeability (48), and PI (40), and the FSC and SSC characteristics (34). We have validated these methods for assessing lymphocyte apoptosis in previous studies (10, 37, 39). No more than 1% of peripheral blood CD4 and CD8 lymphocytes from healthy individuals undergo apoptosis, as determined using these methods (10, 39). We have also found using these techniques a correlation between the stage of HIV infection and the rate of lymphocyte apoptosis (39).

These techniques of assaying apoptosis, however, rely mainly on the final stages of the process of apoptosis. Thus, to verify whether an additional pool of lymphocytes is primed to undergo apoptosis, we investigated also the frequency of $DiOC_6(3)^{low}$ cells that have a disrupted $\Delta\Psi_m$ as current evidence indicates that the disruption of mitochondrial membrane potential is an early and irreversible step in the effector phase of cellular apoptosis (24, 29, 33, 44, 54, 55). The identification of cells with low $\Delta\Psi_m$ allows the identification of an additional pool of lymphocytes that are irreversibly committed to undergo apoptosis even though they did not show the morphological characteristics of apoptotic cells, even when investigated by flow cytometry (24, 29, 33, 44, 54, 55). This approach further confirmed that the addition of L-carnitine to the AZT and DDI regimen had a strong impact on the process of lymphocyte apoptosis compared with the group treated only with those antiretrovirals. Indeed, subjects treated also with L-carnitine had a progressive decline in the frequency of lymphocytes with disrupted mitochondrial potential, but this parameter was substantially unchanged in the group of patients who did not receive L-carnitine and, at the end of the study period, these latter patients had a significantly ($p < 0.0011$) greater frequency of $DiOC_6(3)^{low}$ cells (*i.e.,* lymphocytes irreversibly committed to undergo apoptosis despite still lacking the typical morphology of apoptotic cells) in comparison with patients treated with combined AZT and DDI plus L-carnitine. These findings were paralleled by the decline in the frequency of lymphocytes labeled with DCFH-DA, a fluorochrome that detects H_2O_2 generation (46), in the group treated also with L-carnitine ($p < 0.031$), indicating a lesser degree of oxidant stress in cells from these subjects as compared with patients treated only with antiretrovirals.

We also investigated the expression of endogenous mediators involved in the regulation of cell apoptosis. The frequency of lymphocytes expressing caspase-1, a member of the caspase family that is activated and overexpressed during the process of HIV-mediated apoptosis (5, 19, 20, 50), progressively increased during the treatment with AZT and DDI only but, in turn, decreased during combination therapy with antiretrovirals plus L-carnitine, and a significantly lower frequency of caspase-1-positive lymphocytes was found at the end of the study period in this group compared with subjects who did not receive L-carnitine.

The treatment with L-carnitine was also associated with a progressive increase in the frequency of lymphocytes expressing the p35/cyclin-dependent kinase-5 (cdk-5) regulatory subunit. This was not observed in the group treated only with antiretrovirals, and there was a significant difference between the two groups in the frequency of p35-expressing lymphocytes at the end of the study period. The significance of this finding is unclear and difficult to explain. p35 is a regulatory subunit of cdk-5, a key regulator of cell cycle progression (36, 52). There is evidence that cdk-5 expression is specific to apoptotic cells, and high levels of expression and kinase activity are found in dying cells (1, 56). Cyclin-dependent kinases are considered, indeed, candidate apoptotic effectors given their activation during cell proliferation (51). As the highest levels of cdk-5 activity are expressed in the brain with low or undetectable levels in all other tissues (2, 27, 42) it is likely that the p35/cdk-5 pathway has only a marginal impact on lymphocyte apoptosis. This hypothesis could well be in agreement with the increased lymphocyte expression of such a proapoptotic factor as p35 despite the reduced rate of lymphocyte apoptosis we measured in the group of patients given L-carnitine in addition to AZT and DDI. However, when our results are altogether considered, it appears conceivable that exposure to L-carnitine of HIV-infected subjects treated with AZT and DDI triggers a complex alteration in the intracellular pathways and substrates regulating survival and death of lymphocytes, with an ultimate net effect of a greatly reduced rate of lymphocyte apoptotic death.

Recent investigations have suggested that highly active antiretroviral therapy (HAART) including protease inhibitors can lead to a down-regulation of the Fas–FasL system and a decreased sensitivity to Fas-induced apoptosis (6). The reasons for this discrepancy in our data are unclear and require further investigation. An explanation might be that the potency of the AZT–DDI antiretroviral regimen in suppressing HIV-1 replication is not comparable to that of HAART with protease inhibitors even though the efficacy of the AZT–DDI regimen is well recognized (28). Furthermore, in the study by Bohler and colleagues, a significant reduction in Fas-induced apoptosis was observed also in those subjects who did not achieve a complete viral suppression with HAART (6), but preliminary data from our laboratory have shown that treatment with protease inhibitors had no impact on the expression of the Fas–FasL system and susceptibility of lymphocytes to undergo apoptosis (data not shown).

Our results suggest that the spontaneous process of lymphocyte apoptosis associated with HIV infection can be downmodulated only when the antiretroviral regimen is combined with L-carnitine. Antiretroviral therapy, and probably also regimens including protease inhibitors, is not able to affect the process of lymphocyte apoptosis nor the expression of several markers and endogenous mediators, such as the Fas–FasL system and caspase-1, that transduce the intracellular signals of apoptosis. Furthermore, lymphocytes from subjects who received only antiretrovirals had a greater degree of oxidant stress at the mitochondrial level compared with cells from subjects who were treated also with L-carnitine. The recent observation that HAART appears to increase metabolic demand through some mechanisms independent of its effect on viral burden (7, 31, 32, 49, 53) indirectly adds weight to our data. This effect of antiretroviral therapy may result in elevated oxidant stress that might offset or overwhelm the metabolic benefit of a reduction in viral load. Our results are in agreement with those studies (7, 31, 32, 49, 53). Indeed, in the group of

patients treated only with AZT and DDI, we observed a trend toward an increased frequency of lymphocytes stained by DCFH-DA, a fluorochrome that detects H_2O_2 generation at the mitochondrial level. In contrast, we found that the frequency of DCFH-DA^{high} lymphocytes was significantly decreased in the group treated with AZT and DDI plus L-carnitine. It appears from these data that the addition of L-carnitine to the treatment regimen could have a significant impact on the degree of oxidant stress associated with HIV infection (4, 22) and, possibly, the use of antiretroviral drugs itself (7, 31, 32, 49, 53).

Several mechanisms contribute to the antiapoptotic effect of L-carnitine. First, L-carnitine has been shown to interfere with the transduction of Fas-triggered apoptotic signals and involves the down-modulation of ceramide, a major endogenous mediator of apoptosis, through the inhibition of an acidic sphingomyelinase (8, 16, 37). Additional mechanisms are also implicated as L-carnitine is able to prevent the disruption of mitochondrial membrane potential that occurs early during the effector phase of the apoptotic cell death (9). Furthermore, the antiapoptotic effect of L-carnitine could also be partially mediated by its antidepolarizing action at the mitochondrial level and antioxidant activity according to the demonstration that L-carnitine, when administered *in vivo*, is very effective in inhibiting oxygen radical production (4). The activation of the GH–IGF-1 axis by carnitines is also a probable mechanism (17).

The clinical potential of combining L-carnitine with antiretroviral regimens in order to set down and control the accelerated rate of lymphocyte apoptosis seen in HIV-infected subjects should be further investigated in well designed formal trials.

ABBREVIATIONS

7-AAD, 7-aminoactinomycin D; AIDS, acquired immunodeficiency syndrome; AZT, zidovudine; cdk5, cyclin-dependent kinase-5; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DDI, didanosine; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; FACS, fluorescence activated cell sorter; FITC, fluorescein isothiocyanate; FL, fluorescence; FSC, forward scatter; GH, growth hormone; HAART, highly a ctive antiretroviral therapy; H_2O_2, hydrogen peroxide; HIV, human immunodeficiency virus; IGF, insulin-like growth factor; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PI, propidium iodide; SSC, side scatter; $\Delta\Psi_m$, mitochondrial transmembrane potential.

REFERENCES

1. Ahuja HS, Zhu Y, and Zakeri Z. Association of cyclin-dependent kinase 5 and its activator p35 with apoptotic cell death. *Dev Genet* 21: 258–267, 1997.
2. Alvarez A, Toro R, Caceres A, and Maccioni RB. Inhibition of tau phosphorylating protein kinase cdk5 prevents beta-amyloid-induced neuronal death. *FEBS Lett* 459: 421–426, 1999.
3. Ameisen JC, Estaquier J, Idziorek T, and De Bels F. The relevance of apoptosis to AIDS pathogenesis. *Trends Cell Biol* 5: 27–40, 1995.
4. Badley AD, Pilon AA, Landay A, and Lynch DH. Mechanisms of HIV-associated lymphocyte apoptosis. *Blood* 96: 2951–2964, 2000.
5. Biard-Piechaczyk M, Robert-Hebman V, Richard V, Roland J, Hipskind RA, and Devaux C. Caspase-dependent apoptosis of cells expressing the chemokine receptor CXCR4 is induced by cell membrane-associated human immunodeficiency virus type 1 envelope glycoprotein (gp 120). *Virology* 268: 329–344, 2000.
6. Bohler T, Walcher J, Holzl-Wenig G, Geiss M, Bucholz B, Linde R, and Debatin KM. Early effects of antiretroviral combination therapy on activation, apoptosis and regeneration of T cells in HIV-1-infected children and adolescents. *AIDS* 13: 779–789, 1999.
7. Brinkman K, Smeitink JA, Romijn JA, and Reiss P. Mitochondrial toxicity induced by nucleoside-analogue reverse-transcriptase inhibitors is a key factor in the pathogenesis of antiretroviral-therapy-related lipodystrophy. *Lancet* 354: 1112–1115, 1999.
8. Cifone MG, Alesse E, Di Marzio L, Ruggieri B, Zazzeroni F, Moretti S, Famularo G, Steinberg SM, Vullo E, and De Simone C. Effect of L-carnitine treatment in vivo on apoptosis and ceramide generation in peripheral blood lymphocytes from AIDS patients. *Proc Assoc Am Physicians* 109: 146–153, 1997.
9. Cossarizza A, Mussini C, Mongiardo N, Borghi V, Sabbatini A, De Rienzo B, and Franceschi C. Mitochondria alterations and dramatic tendency to undergo apoptosis in peripheral blood lymphocytes during acute HIV syndrome. *AIDS* 11: 19–26, 1997.
10. Delogu G, Moretti S, Antonucci A, Marcellini S, Masciangelo R, Famularo G, Signore L, and De Simone C. Apoptosis and surgical trauma: dysregulated expression of death and survival factors on peripheral lymphocytes. *Arch Surg* 135: 1141–1147, 2000.
11. De Simone C, Tzantzoglou S, Jirillo E, Marzo A, and Vullo V. L-Carnitine deficiency in AIDS patients. *AIDS* 6: 203–205, 1992.
12. De Simone C, Famularo G, Tzantzoglou S, Trinchieri V, Moretti S, and Sorice F. Carnitine depletion in peripheral blood mononuclear cells from patients with AIDS: effect of oral L-carnitine. *AIDS* 8: 655–660, 1994.
13. De Simone C, Cifone MG, Alesse E, Steinberg SM, Di Marzio L, Moretti S, Famularo G, Boschini A, and Testi R. Cell-associated ceramide in HIV-1-infected subjects. *AIDS* 10: 675–676, 1996.
14. De Simone C, Famularo G, Cifone MG, and Mitsuya H. HIV-1 infection and cellular metabolism. *Immunol Today* 17: 256–258, 1996.
15. Deufel T. Determination of L-carnitine in biological fluids and tissues. *J Clin Chem Clin Biochem* 28: 307–311, 1990.
16. Di Marzio L, Alesse E, Roncaioli P, Muzi P, Moretti S, Marcellini S, Amicosante G, De Simone C, and Cifone MG. Influence of L-carnitine on Fas crosslinking-induced apoptosis and ceramide generation in T cell lines: correlation with its effects on purified acidic and neutral sphin-

gomyelinases in vitro. *Proc Assoc Am Physicians* 109, 154–163, 1997.
17. Di Marzio L, Moretti S, D'Alò S, Zazzeroni F, Marcellini S, Smacchia C, Alesse E, Cifone MG, and De Simone C. Acetyl-L-carnitine administration increases insulin-like growth factor 1 levels in asymptomatic HIV-1-infected subjects: correlation with its suppressive effect on lymphocyte apoptosis and ceramide generation. *Clin Immunol* 92: 103–110, 1999.
18. Dive C, Gregory CD, Phipps DJ, Evans DL, Milner AD, and Wyllie AH. Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. *Biochim Biophys Acta* 1133: 275–284, 1992.
19. Enari M, Hug H, and Nagata S. Involvement of an ICE-like protease in Fas-mediated apoptosis. *Nature* 375: 78–81, 1995.
20. Enari M, Talanian RV, Wong WW, and Nagata S. Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. *Nature* 380: 723–726, 1996.
21. Famularo G, De Simone C, Tzantzoglou S, and Trinchieri V. Apoptosis, anti-apoptotic compounds and TNF-α release. *Immunol Today* 15, 495–496, 1994.
22. Famularo G, Moretti S, Marcellini S, Alesse E, and De Simone C. Cellular dysmetabolism: the dark side of HIV infection. *J Clin Lab Immunol* 48: 123–132, 1996.
23. Famularo G, Moretti S, Marcellini S, Trinchieri V, Tzantzoglou S, Santini G, Longo A, and De Simone C. Acetyl-carnitine deficiency in AIDS patients with neurotoxicity on treatment with antiretroviral nucleoside analogues. *AIDS* 11: 185–190, 1997.
24. Ferri KF, Jacotot E, Blanco J, Este JA, Zamzami N, Susin SA, Xie Z, Brothers G, Reed JC, Penninger JM, and Kroemer G. Apoptosis control in syncytia induced by the HIV type 1-envelope glycoprotein complex. Role of mitochondria and caspases. *J Exp Med* 192: 1081–1092, 2000.
25. Gougeon ML and Montagnier L. Apoptosis in AIDS. *Science* 260: 1269–1270, 1993.
26. Gougeon ML, Lecoeur H, Dulioust A, Enouf MG, Crouvoiser M, Goujard C, Debord T, and Montagnier L. Programmed cell death in peripheral lymphocytes from HIV-infected persons. *J Immunol* 156: 3509–3520, 1996.
27. Hayashi T, Warita H, Abe K, and Itoyama Y. Expression of cyclin-dependent kinase 5 and its activator p35 in rat brain after middle cerebral artery occlusion. *Neurosci Lett* 265: 37–40, 1999.
28. HIV Trialists' Collaborative Group. Zidovudine, didanosine, and zalcitabine in the treatment of HIV infection: meta-analyses of the randomised evidence. *Lancet* 353: 2014–2025, 1999.
29. Jacotot E, Ravagnan L, Loeffler M, Ferri KF, Vieira HL, Zamzami N, Costantini P, Druillennec S, Hoebeke J, Briand JP, Irinopoulou T, Daugas E, Susin SA, Cointe D, Xie ZH, Reed JC, Roques BP, and Kroemer G. The HIV-1 viral protein R induces apoptosis via a direct effect on the mitochondrial permeability transition pore. *J Exp Med* 191: 33–46, 2000.
30. Jaworowski A and Crowe SM. Does HIV cause depletion of CD4+ T cells *in vivo* by the induction of apoptosis? *Immunol Cell Biol* 77: 90–98, 1999.
31. Kakuda TN. Pharmacology of nucleoside and nucleotide reverse transcriptase inhibitor-induced mitochondrial toxicity. *Clin Ther* 22: 685–708, 2000.
32. Kakuda TN, Brundage RC, Anderson PL, and Fletcher CV. Nucleoside reverse transcriptase inhibitor-induced mitochondrial toxicity as an etiology for lipodystrophy. *AIDS* 13: 2311–2312, 1999.
33. Kroemer G, Zamzami N, and Susin SA. Mitochondrial control of apoptosis. *Immunol Today* 18: 44–51, 1997.
34. Lecoeur H, Ledru E, and Gougeon ML. A cytofluorometric method for the simultaneous detection of both intracellular and surface antigens of apoptotic peripheral lymphocytes. *J Immunol Methods* 217: 11–26, 1998.
35. Liegler TJ, Yonemoto W, Elbeik T, Vittinghoff E, Buchbinder SP, and Greene WC. Diminished spontaneous apoptosis in lymphocyte from human immunodeficiency virus-infected long-term nonprogressors. *J Infect Dis* 178: 669–679, 1998.
36. Meyerson M, Enders GH, Wu CL, Su LK, Gorka C, Nelson C, Harlow E, and Tsai LH. A family of human cdc2-related protein kinases. *EMBO J* 11: 2909–2117, 1992.
37. Moretti S, Alesse E, Di Marzio L, Zazzeroni F, Ruggeri B, Marcellini S, Famularo G, Steinberg SM, Boschini A, Cifone MG, and De Simone C. Effect of L-carnitine on human immunodeficiency virus-1 infection-associated apoptosis: a pilot trial. *Blood* 91: 3817–3824, 1998.
38. Moretti S, Alesse E, Marcellini S, Di Marzio L, Zazzeroni F, Parroni R, Famularo G, Boschini A, Cifone MG, and De Simone C. Combined antiviral therapy reduces HIV-1 plasma load and improves CD4 counts but does not interfere with ongoing lymphocyte apoptosis. *Immunopharmacol Immunotoxicol* 21: 645–665, 1999.
39. Moretti S, Marcellini S, Boschini A, Famularo G, Santini G, Alesse E, Steinberg SM, Cifone MG, Kroemer G, and De Simone C. Apoptosis and apoptosis-associated perturbations of peripheral blood lymphocytes during HIV infection: comparison between AIDS patients and long-term non-progressors. *Clin Exp Immunol* 122: 364–373, 2000.
40. Nicoletti I, Migliorati G, Pagliacci C, Grignani F, and Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* 139: 271–279, 1968.
41. Novelli GP, Adembri C, Brunelleschi S, Livi P, Rossi P, and Pratesi C. Oxygen-radicals production during ischemia–reperfusion of the lower limbs in man: inhibitory effects of L-carnitine. *Curr Ther Res* 48: 903–907, 1990.
42. Patrick GN, Zukerberg L, Nikolic M, de la Monte S, Dikkes P, and Tsai LH. Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. *Nature* 402: 615–622, 1999.
43. Petit PX, O'Connor JE, Grunwald D, and Brown SC. Analysis of the membrane potential of rat- and mouse-liver mitochondria by flow cytometry and possible applications. *Eur J Biochem* 194: 389–395, 1990.
44. Petit PX, Lecoeur H, Zoen F, Dauguet C, Mignotte B, and Gougeon ML. Alteration in mitochondrial structure and function are early events of dexamethasone-induced thymocyte apoptosis. *J Cell Biol* 130: 157–167, 1995.
45. Plymale DR, Tang DS, Comardelle AM, Fermin CD, Lewis DE, and Garry RF. Both necrosis and apoptosis contribute

to HIV-1-induced killing of CD4 cells. *AIDS* 13: 1827–1839, 1999.
46. Rothe G and Valet G. Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2′,7′-dichlorofluorescin. *J Leukoc Biol* 47: 440–446, 1990.
47. Sailaja G, Nayak R, and Antony A. Azidothymidine induces apoptosis in mouse myeloma cell lines Sp 2/0. *Biochem Pharmacol* 52: 857–862, 1996.
48. Schmid L, Uittenbogaart CH, Keld B, and Giorgi JV. A rapid method for measuring apoptosis and dual-color immunofluorescence by single laser flow cytometry. *J Immunol Methods* 170: 145–157, 1994.
49. Shevitz AH, Knox TA, Spiegelman D, Roubenoff R, Gorbach SL, and Skolnik PR. Elevated resting energy expenditure among HIV-seropositive persons receiving highly active antiretroviral therapy. *AIDS* 13: 1351–1357, 1999.
50. Stewart SA, Poon B, Song JY, and Chen IS. Human immunodeficiency virus type 1 vpr induces apoptosis through caspase activation. *J Virol* 74: 3105–3111, 2000.
51. Tang D and Wang JH. Cyclin-dependent kinase 5 (Cdk5) and neuron-specific Cdk5 activators. Prog Cell Cycle Res 2: 205–216, 1996.
52. Tsai LH, Delalle I, Caviness VS, Chae T, and Harlow E. p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5. Nature 371: 419–423, 1994.
53. Walker UA, Setzer B, and Volksbeck SI. Toxicity of nucleoside-analogue reverse-transcriptase inhibitors. *Lancet* 355: 1096, 2000.
54. Zamzami N, Marchetti P, Castedo M, Zanin C, Vayssiere JL, Petit PX, and Kroemer G. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. *J Exp Med* 181: 1661–1672, 1995.
55. Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, Susin SA, Petit PX, Mignotte B, and Kroemer G. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *J Exp Med* 182: 367–377, 1995.
56. Zhang Q, Ahuja HS, Zakeri ZF, and Wolgemuth DJ. Cyclin-dependent kinase 5 is associated with apoptotic cell death during development and tissue remodeling. Devel Biol 183: 222–233, 1997.

Address reprint requests to:
Dr. Giuseppe Famularo
Department of Medical Sciences
Ospedale San Camillo
Circonvallazione Gianicolense
00152 Rome, Italy

E-mail: gfamularo@uni.net

Received for publication June 22, 2001; accepted September 21, 2001.

This article has been cited by:

1. Vittorio Calabrese, Carolin Cornelius, Albena T. Dinkova-Kostova, Ivo Iavicoli, Rosanna Di Paola, Aleardo Koverech, Salvatore Cuzzocrea, Enrico Rizzarelli, Edward J. Calabrese. 2011. Cellular stress responses, hormetic phytochemicals and vitagenes in aging and longevity. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* . [CrossRef]
2. Janine Keller, Robert Ringseis, Steffen Priebe, Reinhard Guthke, Holger Kluge, Klaus Eder. 2011. Dietary L-carnitine alters gene expression in skeletal muscle of piglets. *Molecular Nutrition & Food Research* **55**:3, 419-429. [CrossRef]
3. El-Sayed E. El-Awady, Yasser M. Moustafa, Dina M. Abo-Elmatty, Asmaa Radwan. 2011. Cisplatin-induced cardiotoxicity: Mechanisms and cardioprotective strategies. *European Journal of Pharmacology* **650**:1, 335-341. [CrossRef]
4. Neera Singhal, Dean Fergusson, Harold Huff, Edward J. Mills, Charles la Porte, Sharon Walmsley, D. William Cameron. 2010. Design and methods of the MAINTAIN study: A randomized controlled clinical trial of micronutrient and antioxidant supplementation in untreated HIV infection. *Contemporary Clinical Trials* **31**:6, 604-611. [CrossRef]
5. Asma Ahmed, Sambuddho Mukherjee, Mukta Deobagkar, Tanushree Naik, Dipankar Nandi. 2010. Rapid burst of H2O2 by plant growth regulators increases intracellular Ca2+ amounts and modulates CD4+ T cell activation. *International Immunopharmacology* **10**:11, 1397-1405. [CrossRef]
6. N Calandrella, C De Seta, G Scarsella, G Risuleo. 2010. Carnitine reduces the lipoperoxidative damage of the membrane and apoptosis after induction of cell stress in experimental glaucoma. *Cell Death and Disease* **1**:8, e62. [CrossRef]
7. Mehmet Kanter, Yeter Topcu-Tarladacalisir, Sule Parlar. 2010. Antiapoptotic effect of l-carnitine on testicular irradiation in rats. *Journal of Molecular Histology* **41**:2-3, 121-128. [CrossRef]
8. Virginia L. Scofield, Mingshan Yan, Xianghong Kuang, Soo-Jin Kim, Derek Crunk, Paul K.Y. Wong. 2009. The drug monosodium luminol (GVT®) preserves thymic epithelial cell cytoarchitecture and allows thymocyte survival in mice infected with the T cell-tropic, cytopathic retrovirus ts1. *Immunology Letters* **122**:2, 159-169. [CrossRef]
9. Sedat Ustundag, Saniye Sen, Omer Yalcin, Senturk Ciftci, Bora Demirkan, Mevlut Ture. 2009. L-Carnitine Ameliorates Glycerol-Induced Myoglobinuric Acute Renal Failure in Rats. *Renal Failure* **31**:2, 124-133. [CrossRef]
10. Jose L. Miguel-Carrasco, Alfonso Mate, Maria T. Monserrat, Jose L. Arias, Oscar Aramburu, Carmen M. Vázquez. 2008. The Role of Inflammatory Markers in the Cardioprotective Effect of L-Carnitine in L-NAME-Induced Hypertension. *American Journal of Hypertension* **21**:11, 1231-1237. [CrossRef]
11. Orhan Sezen, Mustafa Vecdi Ertekin, Berna Demircan, #hsan Karsl#o#lu, Fazl# Erdo#an, #brahim Koçer, #lknur Çal#k, Akçahan Gepdiremen. 2008. Vitamin E and l-carnitine, separately or in combination, in the prevention of radiation-induced brain and retinal damages. *Neurosurgical Review* **31**:2, 205-213. [CrossRef]
12. T THANGASAMY, M SUBATHRA, S SITTADJODY, P JEYAKUMAR, A JOYEE, E MENDOZA, P CHINNAKKANU. 2008. Role of l-carnitine in the modulation of immune response in aged rats. *Clinica Chimica Acta* **389**:1-2, 19-24. [CrossRef]
13. Rebecca Saich, Clare Selden, Myrddin Rees, Humphrey Hodgson. 2007. Characterization of Pro-apoptotic Effect of Liver Failure Plasma on Primary Human Hepatocytes and Its Modulation by Molecular Adsorbent Recirculation System Therapy. *Artificial Organs* **31**:9, 732-742. [CrossRef]
14. Ibrahim Kocer, Seyithan Taysi, Mustafa Vecdi Ertekin, Ihsan Karslioglu, Akcahan Gepdiremen, Orhan Sezen, Korkmaz Serifoglu. 2007. The effect of L-carnitine in the prevention of ionizing radiation-induced cataracts: a rat model. *Graefe's Archive for Clinical and Experimental Ophthalmology* **245**:4, 588-594. [CrossRef]

15. L GOMEZAMORES, A MATE, E REVILLA, C SANTAMARIA, C VAZQUEZ. 2006. Antioxidant activity of propionyl-l-carnitine in liver and heart of spontaneously hypertensive rats. *Life Sciences* **78**:17, 1945-1952. [CrossRef]
16. Harun ÜÇÜNCÜ, Mustafa Vecdi ERTEKIN, Özgür YÖRÜK, Orhan SEZEN, Asuman ÖZKAN, Fazlı ERDOĞAN, Ahmet KıZıLTUNÇ, Cemal GÜNDOĞDU. 2006. Vitamin E and L-carnitine, Separately or in Combination, in The Prevention of Radiation-induced Oral Mucositis and Myelosuppression: a Controlled Study in A Rat Model. *Journal of Radiation Research* **47**:1, 91-102. [CrossRef]
17. L. Gómez-Amores, A. Mate Barrero, E. Revilla Torres, C. Santa-María Pérez, C.M. Vázquez Cueto. 2005. El tratamiento con propionil-L-carnitina mejora el estrés oxidativo asociado a la hipertensión arterial. *Hipertensión* **22**:3, 109-116. [CrossRef]
18. Catherine L. Cherry, Luxshimi Lal, Steven L. Wesselingh. 2005. Mitochondrial toxicity of nucleoside analogues: mechanism, monitoring and management. *Sexual Health* **2**:1, 1. [CrossRef]
19. GIUSEPPE FAMULARO, CLAUDIO SIMONE, VITO TRINCHIERI, LUCIANA MOSCA. 2004. Carnitines and Its Congeners: A Metabolic Pathway to the Regulation of Immune Response and Inflammation. *Annals of the New York Academy of Sciences* **1033**:1, 132-138. [CrossRef]
20. IRINI MANOLI, MASSIMO U. MARTINO, TOMOSHIGE KINO, SALVATORE ALESCI. 2004. Modulatory Effects of l-Carnitine on Glucocorticoid Receptor Activity. *Annals of the New York Academy of Sciences* **1033**:1, 147-157. [CrossRef]
21. Andrea Cossarizza, Graeme Moyle. 2004. Antiretroviral nucleoside and nucleotide analogues and mitochondria. *AIDS* **18**:2, 137-151. [CrossRef]