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Prednisone increases apoptosis in *in vitro* activated human peripheral blood T lymphocytes

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SUMMARY

Glucocorticoid hormones (GCH) regulate, through the apoptotic process, the negative selection of immature T cells in the thymus. Because apoptosis seems to occur also in the maintenance of peripheral tolerance, we have investigated whether GCH may induce apoptosis in human mature lymphocytes. Peripheral blood lymphocytes (PBL) or peripheral $CD4^+$ and $CD8^+$ T cell subsets were cultured in the presence of phytohaemaglutinin (PHA) or PHA and prednisone (PDN) at 10^{-3}–10^{-12} M concentrations for 72, 96 and 120 h. Cell cycle and membrane antigen expression were evaluated by flow cytometry and DNA degradation was detected by agarose gel electrophoresis. PDN blocks PBL growth in the G_1 phase of cell cycle and inhibits both IL-2 receptor (IL-2R) expression and IL-2 secretion. Apoptosis is clearly increased by PDN in PHA-activated human PBL, and the apoptotic effect of PDN is stronger on $CD8^+$ than on $CD4^+$ T lymphocytes. All these effects are dose- and time-dependent. The addition of exogenous IL-2 did not rescue lymphocytes from PDN-increased apoptosis. These results show that PDN increases apoptosis in mature activated human peripheral blood lymphocytes, suggesting a possible role of GCH in the maintenance of immune tolerance at post-thymic level.

Keywords prednisone apoptosis T lymphocytes cytokines glucocorticoids

INTRODUCTION

Glucocorticoid hormones (GCH) are included among the most potent immunosuppressive drugs, since they affect several effector phases of the immune response. Indeed, the activity of GCH involves the inhibition of the antigen presentation process [1], the production of IL-2 and interferon-gamma (IFN-γ) [2], as well as the synthesis and surface expression of several membrane-bound proteins, including the MHC gene products [3] and the receptors for several cytokines [4].

Apoptosis has been shown as the basic mechanism for deletion of thymocytes following T cell receptor $(TCR)^-$ antigen induction [5]. Moreover, in the thymus, GCH seem to regulate the negative selection of immature T cells inducing thymocyte apoptosis [6]. Recently, it has been reported that apoptosis may also occur in mature activated mouse T lymphocytes [7], and a possible role of apoptosis in the maintenance of peripheral tolerance has therefore been suggested. Moreover, it has been shown that GCH induce apoptosis in murine Th1 and Th2 cell clones, in human T cell hybridomas [8,9] and in lymphoblastoid T cell lines derived from human peripheral blood lymphocytes (PBL) [10]. Lastly, GCH both down regulate IL-2 production and inhibit the signal transduction through the IL-2 receptor (IL-2R) in human T cells [11].

Another intriguing question concerns the influence of cytokines, such as IL-2, in the apoptotic process. In fact, exogenous IL-2 rescues T lymphocytes from GCH-induced cell death [12], but can induce apoptosis in different experimental conditions [13].

In order to investigate whether GCH may induce apoptosis in human mature lymphocytes, the effect of prednisone (PDN) on resting and activated PBL as well as on activated $CD4^+$ and $CD8^+$ T lymphocyte subpopulations was studied. PDN increased apoptosis in mature activated PBL, and this effect was stronger on $CD8^+$ than on $CD4^+$ T lymphocytes. The apoptotic effect of PDN on human activated lymphocytes was not counteracted by exogenous IL-2.

MATERIALS AND METHODS

Cells

PBL were isolated from heparinized blood of 10 healthy

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subjects (age 20–40 years) by Ficoll–Hypaque gradient centrifugation. PBL were washed three times with Hanks' balanced salt solution (HBSS; Eurobio, Paris, France) and resuspended in RPMI 1640 medium (Sigma Chemical Co., St Louis, MO) supplemented with 2 mM L-glutamine, 10% fetal calf serum (FCS), 100 μg/ml streptomycin, and 100 U/ml penicillin (complete medium). PBL were depleted of macrophages upon two consecutive 45-min incubations at 37°C in culture flasks. T cell subpopulations were obtained from PBL by negative selection using Dynabeads M-450 (Dynal Inc., Oslo, Norway) coated with a MoAb specific for the CD4 or CD8 membrane antigens, respectively. Briefly, PBL were resuspended in PBS plus 0·1% FCS at a concentration of 1×10^7 cells/ml. To deplete $CD4^+$ T lymphocytes from PBL, Dynabeads M-450 CD4 were added according to the manufacturer's protocol and incubated for 30 min at 4°C under gentle rotation. The particles were removed using a Dynal magnetic particle concentrator, and a depletion of 99% as assessed by flow cytometric analysis was usually obtained. The same method was used to deplete $CD8^+$ T lymphocytes using Dynabeads M-450 CD8.

Scheme of the experiments

PBL (1×10^6 cells/ml in complete medium) were cultured in 24-well plates at 37°C in a 5% CO_2 atmosphere up to 144 h. PBL cultures were performed in presence of phytohaemagglutinin (PHA; 10 μg/ml; Difco, Detroit, MI) (PBL–PHA) or in presence of PHA and PDN 10^{-3} M, 10^{-6} M, 10^{-9} M and 10^{-12} M (Lepetit MMD, Milan, Italy) added at the beginning of the culture period (PBL–PHA + PDN). In some experiments, PBL were also cultured in presence of PDN alone as control. Analogous experiments were performed using $CD4^+$- or $CD8^+$-enriched T lymphocytes. In some experiments, IL-2 (10 U/ml; Eurocetus, Amsterdam, The Netherlands) was added to PBL–PHA or PBL–PHA + PDN cultures at the beginning of the culture period.

In vitro *PBL proliferation*

The proliferative capacity of cultured PBL was assessed by ^{3}H-thymidine (^{3}H-TdR) incorporation. To this purpose, cultures were pulsed with 10 μCi ^{3}H-TdR (2 Ci/mmol; Amersham, Aylesbury, UK) for the last 8 h of culture. Cultures were stopped at 12, 24, 36, 48, 72, 96, 120, and 144 h after the beginning of the culture period, harvested by an automated device and the radioactivity measured by a beta-counter. Results are expressed as mean ct/min of triplicate experiments.

IL-2 production

To determine the production of IL-2, the supernatants from PBL–PHA or PBL–PHA + PDN cultures, as well as those from cultures set up with $CD4^+$- or $CD8^+$-enriched T lymphocyte populations, were collected after 72, 96 and 120 h of culture and stored at −30°C. IL-2 levels were measured in duplicate by an ELISA assay (Tecnogenetics, Milan, Italy).

Cell cycle and DNA content analysis by flow cytometry

In order to analyse cell cycle and DNA content, 1×10^6 cells were resuspended in 100 μl PBS, lysed, permeabilized, fixed and stained utilizing an automated cell preparator (DNA-PREP EPICS Workstation; Coulter Co., Hialeah, FL). Cellular DNA content was analysed by an Epics Elite flow cytometer (Coulter) after staining the cells with propidium iodide (PI) which binds by intercalation to the double-stranded DNA and emits red fluorescence. In order to recognize DNA diploid peaks, the red fluorescence sensibility of the flow cytometer was set, at the beginning of each analysis, with a reference calibrator which is a suspension of avian erythrocyte nuclei with a constant DNA content. To perform the analysis only on intact single cells, an electronic gate for doublet and clump exclusion was used. To quantify the relative proportions of the various cell populations with the tetraploid, diploid, or hypodiploid DNA content, a software program (Multicycle; Phoenix Flow Systems, San Diego, CA) was used.

To study cell morphology, DNA was stained with PI according to Nicoletti *et al.* [14]. The forward scatter (FS) and the log side scatter (LSS) of particles were simultaneously measured to evaluate the dimension and density of cells.

IL-2R α-chain expression

The percentage of IL-2R α-chain (CD25)-positive cells was measured by cytofluorimetric analysis. The cells were incubated for 30 min at 4°C with the anti-IL-2R α-chain PE-conjugated IL-2R1 MoAb (Coulter), washed twice with PBS–1% FCS, and then analysed with an Epics Elite flow cytometer.

RNA extraction and reverse transcriptase-polymerase chain reaction of GCHR

Total RNA was isolated from resting or activated lymphocytes according to Chomczynski & Sacchi [15] using the RNAzol B method (Biotecx Labs Inc., Houston, TX). Reverse transcription was carried out on 100 ng total RNA in a final volume of 20 μl at 42°C for 1 h using 25 pmol (25 μM) of a 12mer antisense primer downstream of the region to be amplified and 100 U AMV reverse transcriptase (Boehringer-Mannheim, Mannheim, Germany) in polymerase chain reaction (PCR) buffer (10 mM Tris–Cl pH 8·3, 1·5 mM $MgCl_2$, 50 mM KCl, 0·005% Tween 20, 0·005% Nonidet P-40, 10 μg/ml gelatin) containing the deoxynucleotide triphosphates at 10 mM concentration each. Amplification was carried out on the crude cDNA with 2·5 U Taq-DNA-polimerase (Perkin Elmer, Madison, WI) using 25 pmol (2·5 μM) of each of the 24-mer 5′ ACTTCTCTGGGGACTCTGAACTTCC as sense (exon 2, GCHR cDNA sequence), and 24-mer 5′ CCTCTTGA-CAATGGCTTTTCCTAGC (exon 8) as antisense primers in the reaction final volume of 100 μl, and was subjected to 30 cycles at 65°C, 72°C, and 94°C for 30 s each. The amplication product was electrophoresed on 1·4% agarose gel and photographed under UV illumination. A densitometric scanning of reverse transcriptase (RT)-PCR amplification products of GCHR mRNA was then performed. The relative intensity of the bands was determined using a computer program that converts the intensity of each band into arbitrary densitometric units and calculates the peak and the area under the curve.

DNA gel electrophoresis

DNA was isolated by current methodology as described [16]. Briefly, 3×10^6 cells were lysed in TTE solution (Tris–HCl 100 mM, EDTA 10 mM pH 7·4 containing 0·2% Triton X100). DNA fragments were separated from intact chromatin by centrifugation for 10 min at 14926 *g* at 4°C. DNA was precipitated with 5 M NaCl and 1 v ice-cold isopropanol at −20°C overnight. The precipitate was recovered by centrifugation and the pellet washed twice with 75% ethanol. DNA suspended in

TE buffer was treated for 1 h at 37°C with 100 μg/ml RNAse A (Sigma) followed by digestion at 50°C with 250 μg/ml Proteinase K (Boheringer-Mannheim). After a further precipitation, DNA sample in TE buffer was added with loading buffer (10% Ficoll 400, 10 mM EDTA, 0·1% SDS, 0·025% BBF), heated for 10 min at 65°C and immediately electrophoresed. Horizontal electrophoresis was performed for 4 h at 70 V in 1% agarose gel containing 0·5 μg/ml ethidium bromide using TBE (90 mM Tris–HCl, 90 mM boric acid, 2 mM EDTA pH 8) as running buffer.

Electron microscopy

Apoptosis was also evaluated by electron microscopy. Specimens were fixed for 30 min in 2·5% gluteraldehyde in 0·1 M cacodylate buffer and washed in the same buffer. Samples were post-fixed with 1·5% OsO_4 in 0·1% cacodylate buffer, dehydrated in ethanol and embedded in Epon 812 (Epoxy Resins; Shell Chemical Co., Houston, TX). Thin sections, counterstained with uranyl acetate and lead citrate, were observed in a Siemens Elmiscop 101 (Siemens Co., Iselin, NJ).

Statistical analysis

The data obtained with cytofluorimetric analysis are expressed as mean ± s.d., and comparisons among groups were performed by analysis of variance. A two-sided P value $<0·05$ was chosen as significant.

RESULTS

Effect of PDN on PBL proliferation and progression through the cell cycle

PBL proliferation peaked in PHA-activated cultures at the 72nd h of culture (45×10^{-3} ct/min) and was inhibited by PDN 10^{-3} M, 10^{-6} M and 10^{-9} M (9×10^{-3} ct/min, 21×10^{-3} ct/min, and 32×10^{-3} ct/min, respectively).

PHA activation triggers lymphocyte progression from G_1 phase through S and G_2-M phases of the cell cycle. Indeed, in PBL–PHA cultures 34 ● 6%, 25 ± 7% and 24 ± 6% of lymphocytes progressed from G_1 to S phase after 72, 96 and 120 h of culture, respectively. The percentage of cells progressing to S phase in PBL–PHA + PDN 10^{-3} M cultures was significantly lower with respect to PBL–PHA cultures at all times tested ($P < 0·001$). PDN 10^{-6} M reduced the percentage of cells progressing to S phase after 96 h and 120 h of culture only ($P < 0·01$ and $P < 0·001$, respectively), whereas PDN 10^{-9} M and 10^{-12} M did not affect the percentage of cells entering into the S phase at all times of culture (Table 1).

These results suggest that PDN inhibits the progression of PHA-activated PBL through the cell cycle and/or determines the block of cells in G_1 phase in a dose- and time-dependent fashion.

PDN increases apoptosis in PHA-activated PBL cultures

The cytofluorimetric analysis of cell cycle phases demonstrates the presence in PBL–PHA cultures of a low number of hypodiploid cells whose amount slightly increases with culture time, as shown by the sub-G_1 peak on the left side of the histogram (Fig. 1, lane 1). The amount of hypodiploid cells dramatically increases in PBL–PHA + PDN cultures in a time- and dose-dependent fashion, peaking after 120 h of culture and being maximal with PDN 10^{-3} M concentration (Fig. 1, lane 2). The amount of hypodiploid cells in cultures containing PDN 10^{-6} M and 10^{-9} M concentrations was also higher than in PBL–PHA cultures at the same times (Fig. 1, lanes 3 and 4).

In order to calculate the percentage of hypodiploid cells in PBL–PHA + PDN cultures, the Multicycle software program was used. This analysis showed that the percentage of hypodiploid cells significantly increased in PBL–PHA + PDN 10^{-3} M cultures at all times tested ($P < 0·001$), and in PBL–PHA + PDN 10^{-6} M cultures after 96 h and 120 h of culture ($P < 0·05$), and also demonstrated that PDN alone does not induce any modification of DNA content in resting PBL (Fig. 2). Therefore, it seems likely that the apoptotic process triggered by PDN needs the PHA activation of PBL.

In an attempt to explain the apparent resistance of resting PBL to apoptosis, we analysed by RT-PCR whether differences existed in the amount of GCHR mRNA between resting and PHA-activated PBL (Fig. 3a). Densitometric analysis of RT-PCR amplification products of GCHR mRNA showed that the peak (7·29) and the area under the curve (77·63) of PHA-activated PBL are higher than the peak (5·42) and the area under the curve (52·30) of resting PBL, indicating that PHA activation induced an about 50% increase in the amount of GCHR mRNA (Fig. 4).

Early DNA degradation associated with intact cytoplasm cell membrane and modification of cell morphology are classic

Table 1. Cell cycle analysis of peripheral blood lymphocytes (PBL) activated with phytohaemagglutinin (PHA) alone or PHA and prednisone (PDN)*

	Concentration of PDN														
	0			10^{-3} M			10^{-6} M			10^{-9} M			10^{-12} M		
Time (h)	G_1	S	G_2-M	G_1	S	G_2-M	G_1	S	G_2-M	G_1	S	G_2-M	G_1	S	G_2-M
72	65 ± 5†	34 ± 6	6 ± 6	88 ± 3	9 ± 4***	4 ± 2	78 ± 1	28 ± 6	3 ± 3	73 ± 1	33 ± 1	6 ± 5	72 ± 1	32 ± 2	2 ± 2
96	73 ± 7	25 ± 7	5 ± 5	90 ± 5	7 ± 5***	4 ± 3	84 ± 1	13 ± 8**	7 ± 4	66 ± 1	28 ± 1	6 ± 2	73 ± 8	24 ± 1	6 ± 1
120	76 ± 7	24 ± 6	7 ± 2	87 ± 9	11 ± 4***	4 ± 3	87 ± 7	11 ± 3***	8 ± 3	71 ± 1	21 ± 4	7 ± 2	74 ± 1	23 ± 5	9 ± 3

* Percentages of cells in G_1, S or G_2-M phases of the cell cycle after 72, 96 and 120 h of culture with PHA or PHA and PDN.

† Numbers represent the mean ± s.d. of six experiments, and were obtained by flow cytometry utilizing the Multicycle software program (see Materials and Methods).

***$P < 0·001$; **$P < 0·01$ *versus* cultures without PDN.

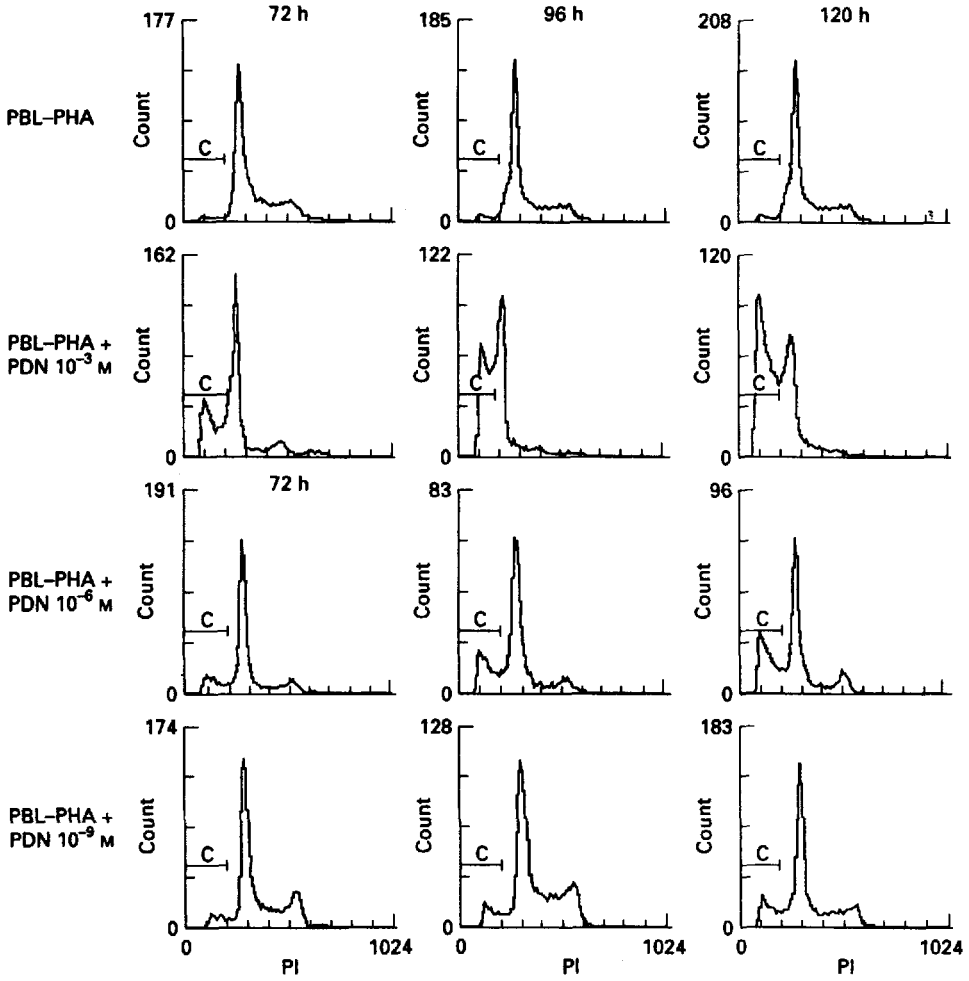

Fig. 1. Cytofluorimetric analysis of cell cycle. The DNA profile of peripheral blood lymphocytes (PBL) cultured with phytohaemagglutinin (PHA) (lane 1) or with PHA and prednisone (PDN) 10^{-3} M, 10^{-6} M and 10^{-9} M (lanes 2, 3 and 4) for 72, 96 and 120 h are shown. Cursor C indicates the hypodiploid DNA content, consistent with the induction of apoptotic nuclear fragmentation. The histograms are representative examples of one out of 10 experiments.

markers of apoptosis. Therefore, lymphocyte morphology of PBL PHA and PBL–PHA + PDN cultures was studied by cytofluorimetric analysis after 96 h of culture. PBL–PHA cultures showed a monomorphic cell distribution due to the homogeneous LSS and FS characteristics of lymphocytes (Fig. 5a). In PBL–PHA + PDN 10^{-3} M cultures two distinct cell populations were detectable (Fig. 5b). The first one, representing the apoptotic cell population, had low FS (due to the reduction of cell volume) and high LSS (due to the condensation of nuclear chromatin) (Fig. 5b, gate I). The presence of hypodiploid DNA in this cell population was confirmed by cytofluorimetric DNA analysis (Fig. 5e). The second one, representing the non-apoptotic cell population (Fig. 5b, gate A) had an euploid DNA content (Fig. 5d). The appearance of this morphologic pattern was time- and dose-dependent. In fact, apoptotic cells in PBL–PHA + PDN 10^{-3} M cultures were detectable already after 72 of culture, and virtually all cells were apoptotic after 120 h of culture (data not shown). Furthermore, the apoptotic cell population was detectable in PBL–PHA + PDN 10^{-6} M cultures after 96 h of culture (Fig. 5c), and few apoptotic cells were also detectable in PBL–PHA + PDN 10^{-9} M cultures at the same time (data not shown).

Electron microscopy showed that cells in PBL–PHA + PDN 10^{-6} M cultures after 96 h of culture had characteristics reported as the morphological changes of apoptotic cell death such as an intact cell membrane, a marginated nuclear chromatin and an initial nuclear fragmentation (Fig. 6a), a marked convolution

Table 2. IL-2 production by peripheral blood lymphocytes (PBL) and T lymphocyte subsets after activation with phytohaemagglutinin (PHA) or PHA and prednisone (PDN)*

	Concentration of PDN				
Cells	0	10^{-3} M	10^{-6} M	10^{-9} M	10^{-12} M
PBL	480	13	30	186	365
$CD4^+$	1000	25	134	250	885
$CD8^+$	600	17	180	350	463

* PBL or $CD4^+$ and $CD8^+$ T lymphocytes were cultured with PHA or PHA and PDN for 96 h. IL-2 concentration in culture supernatants was determined by an ELISA assay. Results are expressed as pg/ml.

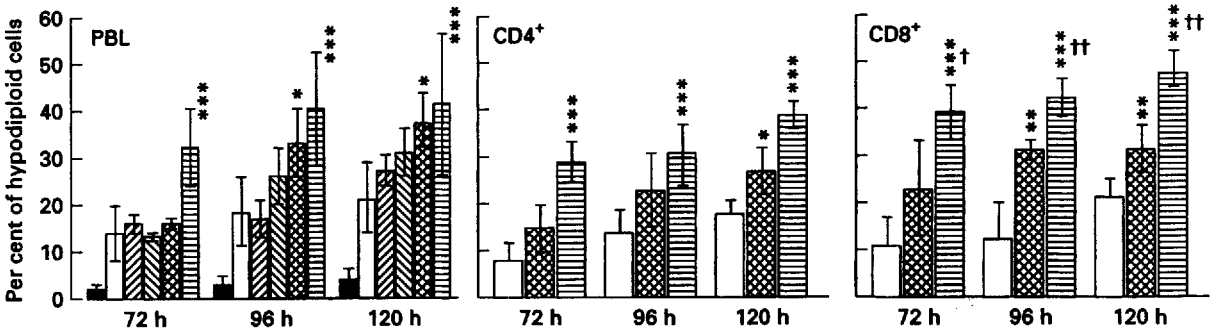

Fig. 2. Percentage of hypodiploid cells in peripheral blood lymphocytes (PBL) and in $CD4^+$ and $CD8^+$ T cells cultured with prednisone (PDN) 10^{-3} M (■), with phytohaemagglutinin (PHA; □) or with PHA and PDN 10^{-3} M (▤), 10^{-6} M (▩), 10^{-9} M (▧) and 10^{-12} M (▨) for 72, 96 and 120 h. Results are the mean ± s.d. of 10 experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, respectively, *versus* cells cultured with PHA; $\dagger P < 0.05$; $\dagger\dagger P < 0.01$, respectively, *versus* $CD4^+$ T cells.

of cellular surface and the development of apoptotic bodies (Fig. 6b).

PDN increases DNA fragmentation in PHA-activated PBL

The intranucleosomal DNA fragmentation, due to a specific endonuclease, leading to a laddered pattern when DNA is analysed by agarose gel electrophoresis, distinguishes apoptosis from necrosis. Fragmented DNA was detectable in PHA-activated PBL when PDN 10^{-3} or 10^{-6} was added to cultures for 120 h (Fig. 7). These data are in agreement with and confirm those obtained by cytofluorimetric analysis.

PDN-increased apoptosis in T lymphocyte subsets

In order to investigate whether any difference in the sensitivity to PDN-induced apoptosis between T lymphocyte subpopulations exists, $CD4^+$- and $CD8^+$-enriched T cell populations were cultured in presence of PHA ($CD4^+$-PHA and $CD8^+$-PHA) or PHA and PDN. A small percentage of apoptotic cells (ranging from 7% to 20%) was detectable both among $CD4^+$-PHA and $CD8^+$-PHA T lymphocytes (Fig. 2). PDN 10^{-3} M significantly increased the percentage of apoptotic cells in both T cell subsets at each culture time ($P < 0.001$), but the percentage of apoptotic cells in $CD8^+$-PHA + PDN 10^{-3} M cultures was significantly higher with respect to $CD4^+$-PHA + PDN 10^{-3} M cultures at each time tested (P values ranging from 0.05 to 0.01). PDN 10^{-6} M induced a significant increase in the percentage of apoptotic cells in $CD8^+$-PHA cultures after 96 h and 120 h of culture ($P < 0.01$) and in $CD4^+$-PHA cultures after 120 h of culture only ($P < 0.05$). Moreover, PDN 10^{-9} M and PDN 10^{-12} M were unable to induce apoptosis in T cell subsets (data not shown).

Agarose gel electrophoresis of low molecular weight DNA extracted from $CD4^+$-PHA and $CD8^+$-PHA cultures showed the presence of oligonucleosomal DNA fragments only in cultures performed in presence of PDN at all times studied. The DNA pattern after 120 h of culture is shown in Fig. 7.

Also for T cell subsets we checked whether the expression of GCHR mRNA was related to apoptosis. As shown in Fig. 3b (lanes 2 and 3), the expression of GCHR mRNA did not differ between $CD4^+$-PHA and $CD8^+$-PHA T lymphocytes.

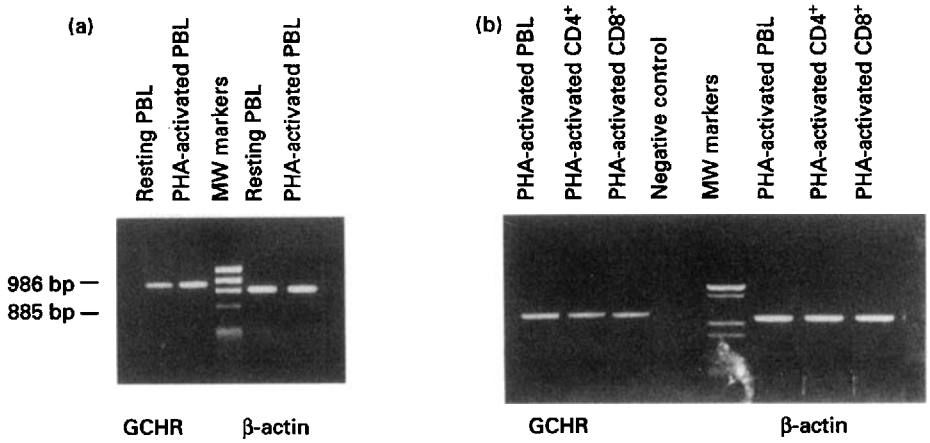

Fig. 3. Reverse transcriptase-polymerase chain reaction (RT-PCR) amplication products of GCHR mRNA. Total RNA was obtained from resting peripheral blood lymphocytes (PBL) (a, lane 1), from phytohaemagglutinin (PHA)-activated PBL (a, lane 2 and b, lane 1) and from PHA-activated $CD4^+$ and $CD8^+$ T lymphocytes (b, lanes 2 and 3). The PCR amplification products of β-actin mRNA are also shown (a, lanes 4 and 5 and b, lanes 6, 7 and 8). RT-PCR reaction carried out in absence of RNA (negative control) is shown (b, lane 4).

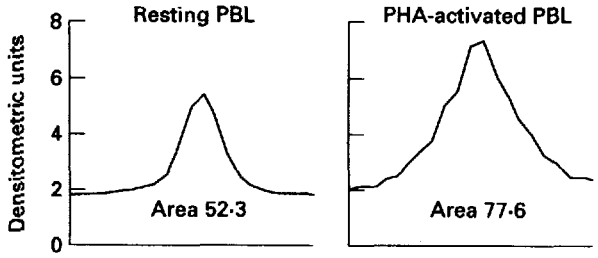

Fig. 4. Densitometric analysis of reverse transcriptase-polymerase chain reaction (RT-PCR) products of GCHR mRNA. The peak and the area under the curve corresponding to resting and phytohaemagglutinin (PHA)-activated peripheral blood lymphocytes (PBL) are shown.

IL-2 production and IL-2R α-chain expression on PHA-activated PBL and T lymphocyte subsets

In the attempt to explain the strong inhibition of PDN on PHA-induced PBL proliferation, we evaluated the amount of IL-2 in supernatants of PBL–PHA, $CD4^+$-PHA and $CD8^+$-PHA cultures. PDN determined a strong inhibition of IL-2 secretion by PBL and T lymphocyte subsets at all times studied, and in a dose-dependent fashion. Data concerning IL-2 secretion after 96 h of culture are reported in Table 2.

The membrane expression of IL-2R α-chain in PBL, $CD4^+$ and $CD8^+$ T lymphocytes following activation with PHA alone or PHA and PDN was also measured. PDN 10^{-3} M induced a significant reduction of the percentage of $CD25^+$ PBL after 72, 96 and 120 h of culture ($P < 0{\cdot}001$), while the lower concentrations of PDN did not affect this parameter, with the exception of PDN 10^{-6} M after 96 h of culture ($P < 0{\cdot}05$) (Fig. 8). PDN 10^{-3} M significantly decreased the expression of IL-2R α-chain also in $CD4^+$- and $CD8^+$-enriched T cells. Interestingly, the expression of IL-2R α-chain was inhibited by PDN more on $CD8^+$ than on $CD4^+$ T cells ($P < 0{\cdot}001$ after 96 h of culture); PDN 10^{-6} M was able to induce a reduction of $CD25^+$ cells in both subsets after 120 h of culture ($P < 0{\cdot}05$) (Fig. 8).

Effects of exogenous IL-2 on PDN-dependent apoptosis of PHA-activated PBL

The above reported data indicate that PDN increases apoptosis and decreases IL-2 production and CD25 expression in PHA-activated human PBL. In order to assess whether exogenous IL-2 might rescue PBL from PDN-induced apoptosis, IL-2 (10 U/ml) was added to PHA–PBL + PDN cultures at the beginning of the culture period. No modifications in the percentage of hypodiploid lymphocytes were observed for each dose of PDN and at all culture times. DNA agarose gel electrophoresis confirmed these data (Fig. 7, lanes 5 and 6).

DISCUSSION

The data reported in this study show that PDN: (i) inhibits the *in vitro* proliferation of human PBL and blocks their progression through the cell cycle following PHA activation; (ii) significantly increases apoptosis in *in vitro* PHA-activated human PBL in a

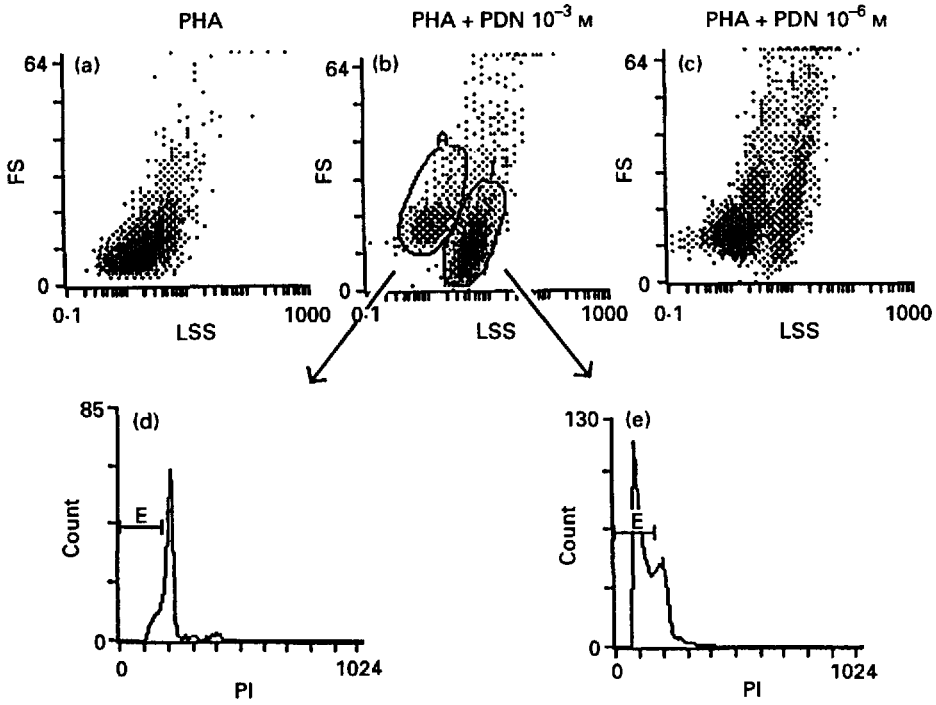

Fig. 5. Cytofluorimetric analysis of cell morphology in peripheral blood lymphocytes (PBL) cultured with phytohaemagglutinin (PHA) (a) or with PHA and prednisone (PDN) 10^{-3} M and 10^{-6} M (b and c, respectively) for 96 h. The propidium iodide (PI) fluorescence of the cell population exhibiting lower log side scatter (LSS) and higher forward angle light scatter (FS) in PHA-activated PBL cultured with PDN 10^{-3} for 96 h (b, gate A) reveals them to be cells with euploid DNA (d). The PI fluorescence of the cell population with an increase in LSS and a decrease in FS (b, gate I) reveals them to be apoptotic cells with hypodiploid DNA (e). The data are a representative example of one out of six experiments.

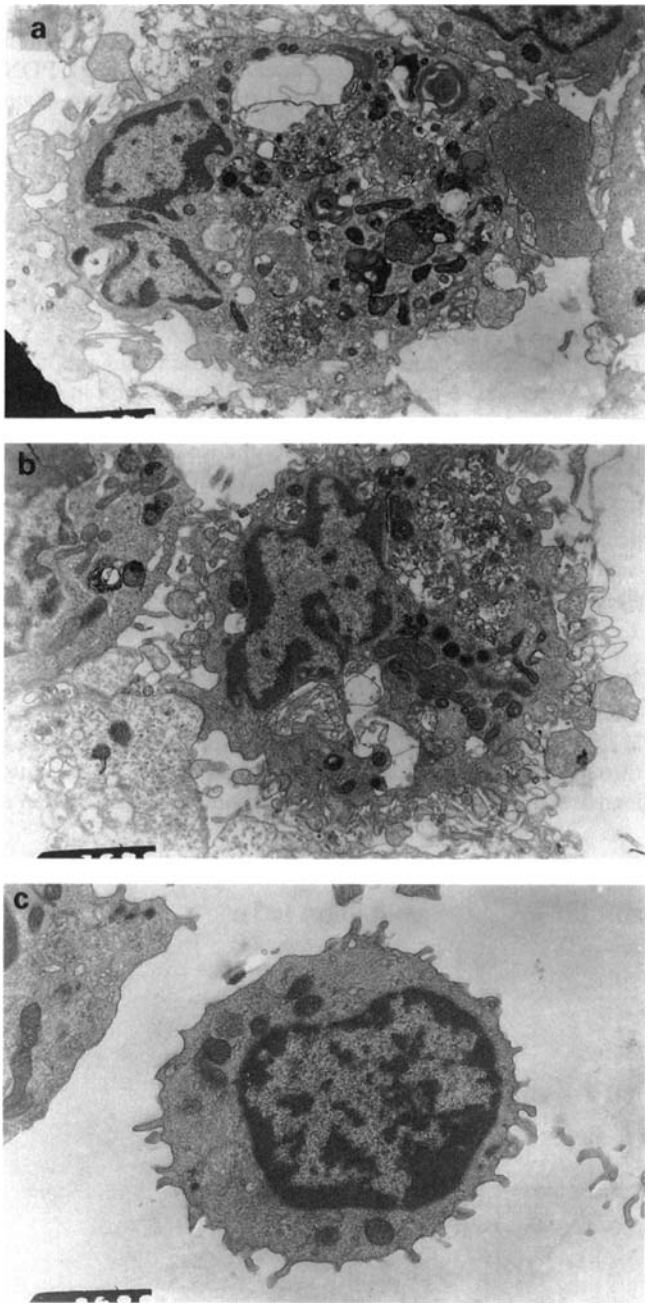

Fig. 6. Electron microscopy of cells in peripheral blood lymphocytes (PBL)–phytohaemagglutinin (PHA) + prednisone (PDN) 10^{-6} M cultures after 96 h of culture. Cells show the morphological characteristics of apoptotic cells such as an intact cell membrane, a marginated nuclear chromatin and an initial nuclear fragmentation (a) as well as a marked convolution of cellular surface and the development of apoptotic bodies (b). A PHA-activated non-apoptotic lymphocyte is shown for comparative purposes (c).

dose- and time-related fashion; (iii) decreases IL-2 secretion and IL-2R expression on PHA-primed human PBL; (iv) affects $CD8^+$ more than $CD4^+$ T lymphocytes. Furthermore, PDN-increased apoptosis is not counteracted by exogenous IL-2 and, finally, most of the above reported effects are detected at an *in vitro* PDN molar concentration (10^{-6} M) that is comparable to its *in vivo* pharmacological range [17].

The inhibition of lymphocyte proliferation and of IL-2 production by PDN confirms analogous observations concerning other GCH [11,18]. The block of lymphocyte progression through the cell cycle is in keeping with the notion that both the accumulation of cells in G_1 compartment and the cessation of cell growth characterize the early phases of apoptosis [19–21]. Whether the inhibitory activities of PDN are exerted by down-regulation of signal transduction through the IL-2R [11], by the defective synthesis of RNA for cytokines needed for T cell proliferation and function [18], or through the interference of the GCHR with transcriptional factors [22], is not clear. Nevertheless, the lack of protective effect of exogenous IL-2 suggests that PDN-induced apoptosis can not be related only to the withdrawal of IL-2.

GCH play a crucial role in the negative selection of immature thymocytes [23] and in the death of lymphoblastoid cell lines [8] or neoplastic T cells [22] by triggering apoptosis. Although it has been reported that GCH may drive activated mature murine PBL into the apoptotic process [10], no data have been reported about the capacity of GCH to trigger apoptosis in human short term cultured polyclonal mature lymphocytes.

We show that the state of activation is a crucial factor for PDN to trigger apoptosis in PBL. The finding that PHA-activated PBL express a higher amount of GCHR mRNA than resting PBL confirms previous data indicating that GCHR increase in activated lymphocytes [24], and may provide an explanation for our observation. It may also correlate with the reported capacity of GCHR to mediate apoptosis by interference with transcriptional factors crucial for cell survival [22].

Although GCH exert their immunomodulatory effects mainly on the $CD4^+$ T subset [1], present data suggest that PDN, at the highest dose tested, induces apoptosis preferentially in $CD8^+$ T cells. This finding may be attributed to the stronger down-regulation of IL-2R expression induced by PDN on $CD8^+$ with respect to $CD4^+$ T cells, and should not be attributed to differences in GCHR level, as GCHR mRNA expression is about the same in both T cell subsets. Such a result is in keeping with our unpublished observation that PDN down-regulates the expression of CD8 more efficiently than that of CD4 molecule, and with the observation that murine $CD4^+/CD8^+$ double-positive thymocytes are driven into the apoptotic process when the level of CD8 and CD4 molecules is very low [25,26], but it is in contrast with the finding that overexpression of CD8 transgenes in thymocytes may result in intrathymic cell death [27,28]. Furthermore, these data disagree with those reported by Wesselborg *et al.* [7], who failed to demonstrate any difference between $CD4^+$ and $CD8^+$ human T cell clone apoptosis triggered *via* the TCR/CD3 complex. These conflicting results might be attributed to the different experimental conditions used in our assays, i.e. the activation of PBL through the CD2 pathway, and to the use of polyclonal cell population.

A final question is whether these findings, even if obtained with pharmacological doses of PDN, might reflect a mechanism operating in physiologic conditions. Since GCH are involved in the control of biologic homeostasis, it is likely that they may trigger apoptosis in PBL when activated by antigens *in vivo*. If this is the case, it could be suggested that GCH-enhanced PBL apoptosis may add to other known mechanisms, such as down-

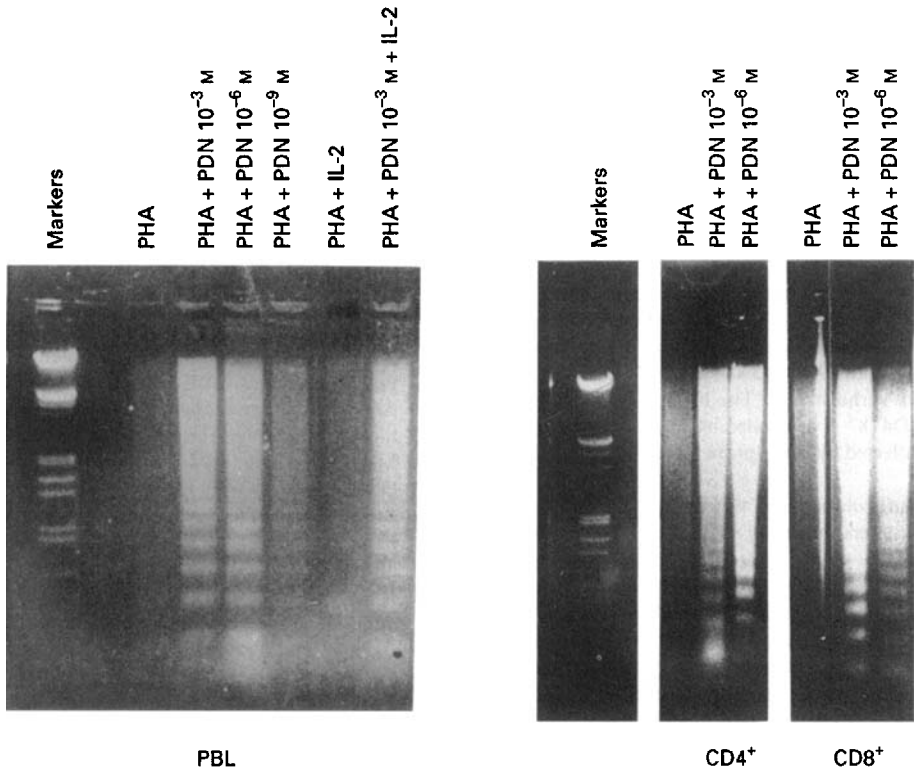

Fig. 7. Agarose gel electrophoresis of low molecular weight DNA extracted from peripheral blood lymphocytes (PBL) and from $CD4^+$ and $CD8^+$ T cells. Cells were cultured with phytohaemagglutinin (PHA), with PHA + prednisone (PDN) 10^{-3} M, 10^{-6} M or 10^{-9} M, with PHA + IL-2 and with PHA + PDN 10^{-3} M + IL-2 for 120 h. The DNA fragmentation pattern characteristic of apoptosis is present in PBL and in $CD4^+$ and $CD8^+$ T cells cultured with PHA + PDN, at all the doses tested, as well as in PBL cultured with PHA + PDN 10^{-3} M + IL-2. Analogous results were obtained in six separate experiments. Markers = λ DNA-EcoRI + Hind III digest fragments.

regulation of TCR expression and induction of anergy [29], in the maintenance of peripheral tolerance. Since the GCH serum level is physiologically regulated by neuropituitary hormones and by several cytokines, GCH might constitute an efferent branch of the immunoneuroendocrine regulatory network controlling the expansion of activated T cell clones.

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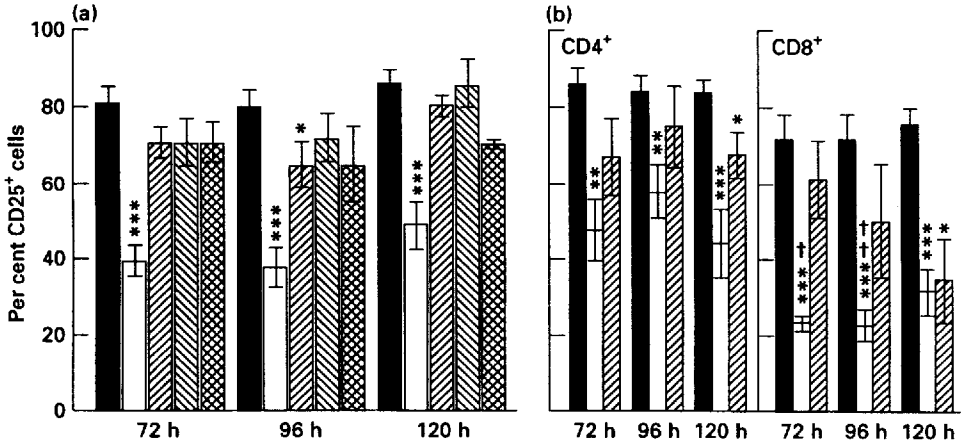

Fig. 8. Percentage of IL-2R α-chain (CD25) positive cells in peripheral blood lymphocytes (PBL) (a) and in $CD4^+$ and $CD8^+$ T cells (b) cultured with phytohaemagglutinin (PHA; ■) or with PHA and prednisone (PDN) 10^{-3} M (□) 10^{-6} M (▨), 10^{-9} M (▧) and 10^{-12} M (▩) for 72, 96 and 120 h. Results are the mean ± s.d. of 10 experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, respectively *versus* cells cultured with PHA; $\dagger P < 0.01$; $\dagger\dagger P < 0.001$ *versus* $CD4^+$ T cells.

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